CCXLIII. THE PREPARATION OF CONCENTRATED ANTIGONADOTROPIC FACTOR (ANTIPROLAN)

BY BERNHARD ZONDEK, FELIX SULMAN AND ABRAHAM HOCHMAN

From the Laboratory of the Obstetrical-Gynaecological Department of the Rothschild Hadassah Hospital, Jerusalem

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THOMPSON [1937] showed that when antigonadotropic sera are salted out with 33–50% sat. $(NH_4)_2SO_4$, the antigonadotropic factor may be quantitatively recovered from the pseudoglobulin fraction. Zondek & Sulman [1937] described independently several methods of preparation of the antigonadotropic factor, viz. precipitation with acetone and salting out with $(NH_4)_2SO_4$ directly from the serum or from a solution of an acetone-powder prepared therefrom. Harington & Rowlands [1937] confirmed and extended these results in an extensive series of experiments.

The following methods of preparation have now been studied by us:

(1) Direct precipitation, utilizing acetone, alcohol, benzoic acid, flavianic acid and tannic acid, both alone and combined.

(2) Fractional precipitation, utilizing $(NH_4)_2SO_4$, KI plus NaCl, precipitation at the isoelectric point, precipitation by CO_2, precipitation by dialysis and combinations of these methods.

(3) Adsorption and elution, utilizing kaolin, Lloyd's adsorbent, aluminium hydroxides A and B prepared as described by Willstätter, "Fasertonerde", franconite and benzoic acid.

(4) Coagulation and elution, utilizing acetone, alcohol and heat.

(1) *Direct precipitation*

(*a*) *Precipitation by acetone.* The method of precipitation by acetone was described by Zondek & Sulman [1937]; 1 mg. of the acetone-powder contains 1–3 P.A.U.[1]), the concentration depending on the titre of the original serum. The loss by precipitation amounts to 10% at the most. This method of preparation renders storage of antigonadotropic factor in the desiccator without loss in titre for more than a year possible. Rabbit antisera treated by this method yield an acetone-powder which dissolves readily in water and normal saline giving concentrations of active substance 3–4 times greater than in blood. The acetone-powder from goat antisera, on the other hand, is difficultly soluble even to the original blood concentration, and its solutions always retain a slight opalescence.

(*b*) *Precipitation by alcohol.* The technique of the alcohol precipitation is the same as that of the acetone method. The results with alcohol are not as good,

[1] 1 P.A.U. = 1 prolan anti-unit is the smallest amount of antiprolan able to neutralize the gonadotropic effect of 1 R.U. of prolan. At least 10 units must be assayed in a test rat [cf. ZONDEK & Sulman, 1937].

however, since the alcohol-powder is only incompletely soluble and the solutions are more opalescent than are those obtained with the acetone-powder.

(*c*) *Precipitation by benzoic acid.* On addition of a concentrated solution of benzoic acid in acetone to antiserum a precipitate is formed. This precipitate can be freed from benzoic acid by rinsing in acetone and the residue dried with ether. The dry powder thus obtained is difficultly soluble in water or normal saline, and is no richer in antiprolan than the material obtained in the simpler direct precipitation with acetone.

(*d*) *Precipitation by flavianic acid.* With a few drops of 10% aqueous flavianic acid maximum precipitation of a 1 : 10 dilution of prolan antiserum is obtained. After 48 hr. in the ice chest, such a precipitate was centrifuged and freed from flavianic acid by washing with 0·5% ammonia in 96% alcohol. The residue was dried by washing first with acetone and then with ether. The dry powder obtained was partly soluble in distilled water. The water-soluble portion (A) was rich in protein but poor in antiprolan, and could be refractionated by salting out with 50% sat. $(NH_4)_2SO_4$ into two components both of which contained but little antiprolan. The water-insoluble portion (B) dissolved in Locke solution (as well as in $N/50$ ammonia and buffers of pH 9·5) giving a solution which was poor in protein, could not be further fractionated with $(NH_4)_2SO_4$ and was no richer than A in antiprolan. Quantitative analysis showed that the flavianic acid precipitated only 50% of the total antiprolan and that this percentage was distributed equally between fractions A and B of the precipitate. The method described is not therefore promising.

(*e*) *Precipitation with tannic acid.* The addition of a few drops of a 10% aqueous tannic acid to prolan antiserum brought about maximal precipitation. The sedimented material proved to be rich in protein as well as in antiprolan, but attempts to separate the two were unsuccessful.

Of the five methods of antiprolan precipitation tested, therefore, the acetone method alone gives satisfactory results.

(2) *Preparation by fractional precipitation*

(*a*) *Fractional precipitation with sat.* $(NH_4)_2SO_4$. The results obtained with this method were described by Zondek & Sulman [1937]. Rabbit serum fibroglobulin (25% sat. $(NH_4)_2SO_4$) and euglobulin (32% sat. $(NH_4)_2SO_4$) fractions were found to be free of antiprolan, the whole of which was contained in the pseudoglobulin (48% sat. $(NH_4)_2SO_4$) fraction; transition fractions (32–48% sat. $(NH_4)_2SO_4$) contained antiprolan only in proportion to their contents of pseudoglobulin.

The same experiments when carried out with goat antiserum gave different results in that with this serum about 50% of the antiprolan was brought down with the euglobulin, and only 50% with the pseudoglobulin fractions [cf. Harington & Rowlands, 1937]; as in the rabbit the fibroglobulin and albumin fractions contained no antiprolan. Our results with salting out in diluted antisera were not promising, but with acetone-powder from rabbit antisera salting out was successful in antiprolan solutions of twice the physiological concentration: at 9·1% sat. $(NH_4)_2SO_4$ a very light precipitate containing no antiprolan separated; at 18·2% no further precipitate was obtained but at 23·1% saturation a heavy inactive precipitate formed; at 28·6% saturation a further light precipitate containing the entire antiprolan content of the antiserum was obtained; further inactive precipitates were obtained at 33·3% saturation. The fractional

$(NH_4)_2SO_4$ precipitation method in acetone-powder solution is effective therefore for concentrating the antigonadotropic factor in rabbit antiserum. Experiments with goat antisera indicated that this method may not give the same favourable results with antisera from other species.

(*b*) *Fractional precipitation with KI plus NaCl.* In 1 ml. prolan antiserum diluted with 2 ml. distilled water was dissolved 1 g. KI; the resulting solution was treated with 1 g. NaCl which did not entirely dissolve. On standing in the ice chest for 24 hr., a light precipitate containing about 50% of the serum antiprolan separated out. The supernatant, after dialysis through a cuprophane membrane, was found to have retained 50% of the total serum antiprolan. The antiprolan yield obtained in this method of precipitation is therefore rather low.

(*c*) *Fractionation by isoelectric precipitation.* The precipitate formed by native rabbit or goat prolan antiserum at the isoelectric point contains practically no antiprolan. By working with solutions poor in electrolytes (solution of acetone-powder in distilled water) and at high dilutions (1 : 25 or 50) and by acidifying gradually with acetic acid, however, a considerable concentration of antigonadotropic factor was possible. Success in the formation of a maximal precipitate at the isoelectric point is chiefly a matter of practice. With experience it is possible to follow the general progress of the precipitation by rough nephelometric estimation. Addition of bromocresol green facilitates both the nephelometric comparison and the determination of the optimum *p*H (approx. 5·5) for precipitation.

Table I shows that the antiprolan content of the isoelectric precipitate depends on the degree of dilution of the solution of acetone-powder.

Table I. *Recovery of antiprolan by isoelectric precipitation of solutions of acetone-powder from rabbit antiserum solution containing 50 mg. powder = 100* P.A.U. *per ml.*

Dilution	Yield of antiprolan in isoelectric precipitate %	Dilution	Yield of antiprolan in isoelectric precipitate %
1 : 1	0	1 : 20	83
1 : 2	33	1 : 25	90
1 : 4	50	1 : 30	90
1 : 8	66	1 : 40	90
1 : 16	75	1 : 50	90

It follows from the table that for a maximal yield of antiprolan in the isoelectric precipitation of rabbit antisera, preliminary dilution of the acetone-powder solution to at least 1 : 25 is desirable. Dilution beyond 1 : 50 is not to be recommended owing to the difficulty of determining the point of optimal precipitation. The isoelectric precipitates obtained are not readily soluble and it may be preferable to take them up in Locke solution. With goat antisera acetone-powder the isoelectric precipitation method has failed as yet to give as favourable results as are given by the rabbit antisera; even at dilutions of 1 : 50, only 50% of the total antiprolan content came down in the isoelectric precipitate. Isoelectric precipitation is therefore an effective method of concentrating antigonadotropic factor, but is not applicable to all species of antiprolan sera. It is specially suited, however, for the purification of rabbit antisera, a concentration to 6 P.A.U. per mg. of dry substance being obtainable.

(*d*) *Fractional precipitation by treatment with CO_2.* On passing CO_2 through antiprolan solutions, precipitation of part of the serum globulins occurs. The centrifuged precipitate contains at best about 40% of the total serum antiprolan. Concentration of antiprolan by this method is therefore impracticable.

(*e*) *Fractional precipitation by dialysis.* Native rabbit antiserum was dialysed in a cuprophane membrane against running tap water for 24 hr. The removal of electrolytes caused partial precipitation of the globulins and with them of 50% of the serum antiprolan. With goat antisera also rapid precipitation of serum globulins containing 50% of the total antiprolan was obtained. Fractional precipitation by dialysis is therefore impracticable as a method of concentrating the antigonadotropic factor.

(*f*) *Combined methods of fractional precipitation.* Two combined methods were tested: method A in which the isoelectric precipitation of rabbit antisera as described under (2 (*c*)) is followed by dialysis of the precipitate dissolved in Locke solution as described under (2 (*e*)) and method B in which the isoelectric precipitate (2 (*c*)) is extracted with 25% sat. $(NH_4)_2SO_4$ (2 (*a*)) instead of with Locke solution.

(i) *Combined method A.* 50 mg. of antiprolan acetone-powder, corresponding to 1 ml. native rabbit serum (=50 P.A.U.), were dissolved in 25 ml. distilled water and brought to maximal turbidity by addition of 1% acetic acid drop by drop in the presence of bromocresol green. After 1 hr. the suspension was centrifuged and the precipitate was taken up in 3 ml. of Locke solution. This was then dialysed in a cuprophane membrane against running tap water for 24 hr. Under this treatment, a hydrophobic fraction of the isoelectric precipitate separated. The isoelectric precipitate without the hydrophobic fraction was found to contain 83% of the total serum antiprolan. 1 mg. of this final material contained 10 P.A.U., the concentration attained being a tenfold one. This purified material when dissolved in 0·005 *N* NaOH and diluted to a concentration of 25 P.A.U. per 4 ml. gave slight ninhydrin and biuret reactions and but a slight clouding with acetone and salicylsulphonic acid; no coagulation occurred on boiling.

(ii) *Combined method B.* 50 mg. antiprolan acetone-powder, corresponding to 1 ml. native rabbit antiserum (=50 P.A.U.), were dissolved in 25 ml. distilled water and brought to maximal turbidity by addition of 1% acetic acid drop by drop in the presence of bromocresol green. After 1 hr. the precipitate was centrifuged, suspended in 3 ml. 25% sat. $(NH_4)_2SO_4$ and shaken for 1 hr. The extract was dialysed against running tap water. A slight inactive precipitate separated. The remaining supernatant liquid contained 75% of the total serum antiprolan at a concentration of 10 P.A.U. per mg. of dry substance, and behaved in protein tests as did the solution obtained in combined method A.

By combined fractional precipitation methods therefore, i.e. (*a*) isoelectric precipitation followed by dialysis, or (*b*) isoelectric precipitation followed by fractionation with sat. $(NH_4)_2SO_4$, a tenfold concentration of the antiprolan content of rabbit antiserum is possible.

(3) *Adsorption and elution*

(*a*) *Adsorption with kaolin and Lloyd's adsorbent.* The adsorbent was added to prolan antiserum at various *p*H values (4·5; 7·0; 9·5) until the reaction of the supernatant fluid to salicylsulphonic acid became negative. The suspensions were centrifuged and filtered and elution by means of glutamic acid, glycine and phosphate buffers at different *p*H values (9·5; 7·0; 4·9) was carried out. Elution of antiprolan from kaolin proved feasible only at alkaline or neutral reaction. Protein and antiprolan in the eluates gave parallel values. Elution from Lloyd's adsorbent failed consistently. These adsorption methods are therefore impracticable as methods of preparation of concentrated antigonadotropic factor.

(*b*) *Adsorption with Willstätter $Al(OH)_3$ A and B.* Antiprolan was adsorbed by alumina in alkaline, neutral and acid solution. Elution with alkaline, acid and neutral buffer solutions was tested. The acid eluents practically dissolved the aluminium compound giving toxic eluates which were useless for the purpose in hand; neutral buffers failed to elute antiprolan whilst alkaline buffers eluted varying amounts but at best not more than 30% of the serum antiprolan. The methods described in this section are therefore of little practical value.

(*c*) *Adsorption with franconite and "Fasertonerde".* Adsorption by these two adsorbents occurred at every *p*H tested. Elution was possible only with alkaline buffers. The protein and antiprolan contents of the eluates gave parallel values, however, and it must be concluded that the method described is impracticable for the preparation of antiprolan in concentrated form.

(*d*) *Adsorption with benzoic acid.* Antiprolan solution was shaken with benzoic acid for 3 hr. The precipitate obtained was centrifuged, freed from benzoic acid by washing three times with acetone and tested for antiprolan. It was found to be inactive.

(4) *Preparation by coagulation and elution*

(*a*) *Coagulation with acetone and elution with dilute acetone.* As Zondek & Sulman have shown [1937], 80% acetone precipitates the antigonadotropic factor. If water is then added to the suspension so that an acetone concentration between 40 and 45% is obtained the antiprolan is eluted in quantities ranging between 33 and 50% of the total available antiprolan. Simultaneously at least 33–50% protein is redissolved. This method is therefore of no value for the preparation of antiprolan concentrates.

(*b*) *Coagulation with alcohol and elution with dilute alcohol.* Use of alcohol as described under 4 (*a*) for acetone gave similar results.

(*c*) *Coagulation by heat and elution with water.* The method here utilized has been used and is recommended by Besredka [1929] for the preparation of antisera concentrates. The antiprolan acetone-powder from rabbit serum was taken up in water in a concentration of 5 : 1. The mash obtained was shaken well and placed in the oven at 65° for 8 hr. to effect coagulation. The coagulate was extracted with distilled water. Since considerable quantities of protein accompanied the eluted antiprolan, such heat-coagulation followed by elution with water is not feasible as a method of separation of the antigonadotropic factor from serum protein.

Discussion

The results presented in this paper show that the antigonadotropic factor cannot be obtained free from globulins by the common methods of adsorption and elution. This property distinguishes the antigonadotropic factor from numerous hormones and in particular from the gonadotropic factor itself. Evans *et al.* [1933], it will be recalled, adsorbed the gonadotropic factor on Willstätter alumina A and B and obtained highly purified hormone preparations on subsequent elution.

The antigonadotropic factor is always contained in the serum globulin, a property which places this factor in the same class as the antibodies. These experiments constitute further support for the view advanced that antiprolan is similar to an antibody and is not a hormone.

SUMMARY

Different methods of concentrating antiprolan are described.

I. Methods which appear to be of little practical value are:

(1) Direct precipitation with alcohol, benzoic acid, flavianic acid or tannic acid.

(2) Fractional precipitation utilizing potassium iodide plus sodium chloride, carbon dioxide or dialysis.

(3) Adsorption and elution, utilizing kaolin, Lloyd's adsorbent, Willstätter alumina A and B, franconite, "Fasertonerde" or benzoic acid.

(4) Coagulation and elution utilizing acetone, alcohol or heat.

II. The methods which were found to give results of significant practical value are:

(5) Precipitation and preservation by treatment with acetone and ether.

(6) Fractional precipitation with saturated ammonium sulphate.

(7) Isoelectric precipitation from dilute solutions poor in electrolytes.

The purest preparations were obtained by combined methods of preparation, viz.:

(8) Combined method A. Isoelectric precipitation of diluted antiserum, followed by solution of the precipitate in Locke solution and dialysis.

Combined method B. Isoelectric precipitation of diluted antiserum, followed by partial solution of the precipitate in 25% sat. $(NH_4)_2SO_4$ and dialysis of the filtrate.

The concentrated antiprolan solutions obtained by the combined methods of preparation gave only a faintly positive reaction in the ninhydrin and biuret tests, developed only slight turbidities with acetone and salicylsulphonic acid and did not coagulate on boiling; 1 mg. purified antiprolan dry substance contained 10 antiprolan units.

The combined methods of preparation were not of equal efficacy with the two species tested, the results obtained with rabbit antiserum by the combined methods being far more promising than those obtained with goat antiserum.

REFERENCES

Besredka (1929). *Kolle-Kraus-Uhlenhuth, Handbuch path. Mikroorgan.* vol. 2, pt. 1, 3rd ed. Bächer, Jena-Berlin-Wien.

Evans, Gustus & Simpson (1933). *J. exp. Med.* **58**, 563.

Harington & Rowlands (1937). *Biochem. J.* **31**, 2049.

Thompson (1937). *Proc. Soc. exp. Biol., N.Y.*, **35**, 640.

Zondek & Sulman (1937). *Proc. Soc. exp. Biol., N.Y.*, **36**, 708, 712; **37**, 193, 198, 343.

CCXLIV. ESTIMATION OF THE ANTI-HAEMORRHAGIC VITAMIN

By H. J. ALMQUIST, E. MECCHI and A. A. KLOSE

From the Division of Poultry Husbandry, University of California, College of Agriculture, Berkeley

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In a former paper [Almquist & Stokstad, 1937] it was stated that chicks fed on diets containing low levels of vitamin K tended to show a maximum blood clotting time at 2 weeks of age and a decreasing blood clotting time after this age. The method of preventive assay and the use of a reference standard, as well as a negative control, were briefly illustrated. Further studies of this assay procedure have been made and may now be reported.

Methods and results

The feeding and housing of the chicks and the determination of blood clotting times were identical with the procedures already described [Almquist & Stokstad, 1937]. In withdrawing blood, incisions about 1 mm. long were made with a fine pointed scalpel in a wing vein which was exposed near the junction of the ulna, radius and humerus.

The sole source of supplementary vitamin K used in these experiments was a hexane extract of alfalfa which has been adopted as a reference standard in our vitamin K assays. 1 ml. represented 1 g. dried alfalfa.

Chicks from hens on diets containing approximately the same vitamin K content were divided into groups and given the basal diet plus various levels of the standard vitamin K sources. The results of one such experiment are given in Table I. The marked tendency toward a maximum blood clotting time at 2 weeks of age is a most striking feature of these results.

Table I. *Average blood clotting times in relation to dietary vitamin K level and age of chicks. Exp. I*

Standard vitamin K soln. per kg. diet (ml.)	No. of chicks	Average blood clotting times in min. at age of			
		1 week	2 weeks	3 weeks	4 weeks
2·0	10	20·2 ± 5·6	Greatly prolonged	10·1 ± 1·2	8·9 ± 0·6
3·0	10	10·3 ± 2·3	19·5 ± 3·0	7·1 ± 0·8	7·0 ± 1·0
4·0	10	6·8 ± 1·0	7·6 ± 0·7	5·8 ± 0·9	4·8 ± 0·6
5·0	10	5·7 ± 0·6	6·3 ± 0·9	5·2 ± 0·6	4·2 ± 0·4
10·0	10	—	Greatly prolonged*	4·2 ± 0·8	4·0 ± 0·5
50·0	10	3·4 ± 0·4	3·5 ± 0·4	3·4 ± 0·4	2·7 ± 0·3

* This group had received only the basal diet for 2 weeks; all individuals were markedly deficient. They received the vitamin K supplement at an age of 2 to 4 weeks.

In a second experiment of this nature we obtained chicks from hens receiving diets rich in vitamin K. Day-old chicks from this source had an average blood clotting time of 1·9 min. as compared with 3·2 min. for chicks from the sources used in the first experiment. These chicks with a high reserve of vitamin K did

not show such a marked increase in blood clotting time when they received less vitamin K in the diet as those of the first experiment, although there was a definite increase during the second week. Apparently, the high vitamin K reserve of these chicks was sufficient to compensate for the low vitamin K intake through the critical period up to 2 weeks of age. The results are given in Table II.

Table II. *Average blood clotting times in relation to vitamin K level and age of chicks. Exp. II*

Standard vitamin K soln. per kg. diet (ml.)	No. of chicks	Average blood clotting time in min. at age of			
		1 week	2 weeks	3 weeks	4 weeks
2·0	16	5·2 ± 0·8	9·6 ± 1·9	10·9 ± 1·7	8·0 ± 1·1
2·5	16	3·5 ± 0·6	8·3 ± 1·2	9·4 ± 1·2	—
3·0	15	3·4 ± 0·2	7·2 ± 1·1	7·8 ± 1·4	—
4·0	15	2·4 ± 0·2	6·6 ± 1·2	6·9 ± 0·9	6·5 ± 0·3
5·0	15	2·6 ± 0·2	5·1 ± 0·5	4·0 ± 0·3	—
10·0	15	2·7 ± 0·3	3·0 ± 0·3	3·0 ± 0·2	—
50·0	15	2·4 ± 0·3	3·9 ± 0·3	3·2 ± 0·2	—
0·0	6	—	Greatly prolonged	—	—

In a third experiment chicks from the same source as in Exp. I were used with a repetition of the results of Exp. I. There was again the well-defined tendency toward a maximum blood clotting time at 2 weeks of age, with many individuals of the lowest vitamin K intake having clotting times that were greatly prolonged. About 15 chicks per group were used in the last two experiments.

The blood clotting times of chicks at 3 weeks of age in these experiments are given in Table III. It is apparent that, although certain groups of chicks in

Table III. *Average blood clotting times at 3 weeks of age of chicks in Exps. I, II and III*

Standard vitamin K soln. per kg. diet (ml.)	Average blood clotting time in min.		
	Exp. I	Exp. II	Exp. III
2·0	10·1 ± 1·2	10·9 ± 1·7	12·5 ± 1·4
2·5	—	9·4 ± 1·2	8·6 ± 0·8
3·0	7·1 ± 0·8	7·8 ± 1·4	7·0 ± 0·8
4·0	5·8 ± 0·9	6·9 ± 0·9	5·7 ± 0·5
5·0	5·2 ± 0·6	4·0 ± 0·3	4·8 ± 0·6
10·0	4·2 ± 0·8	3·0 ± 0·2	4·1 ± 0·2
50·0	3·4 ± 0·4	3·2 ± 0·2	—

Exps. I and III had passed through a period of markedly prolonged blood clotting time and those of Exp. II had not done so, all groups at 3 weeks of age had approximately the same blood clotting time for the same vitamin K level in the diet. It appears that at this age the chicks had achieved a balance with respect to the vitamin K content of the diet.

During Exp. III, three groups of chicks, all with greatly prolonged individual clotting times, were given at 2 weeks of age diets containing 3 ml., 5 ml. and 10 ml. of the standard solution per kg. The average clotting times for these groups at 3 weeks of age were, respectively, 6·9, 5·2 and 3·8 min., while the average clotting times of contemporary groups which had received these vitamin K levels continuously from 1 day of age, were, respectively, 7·0, 4·8 and 4·1 min. These results show that, in spite of a previous condition of severe depletion, the chicks were able, within 1 week or less, to attain an average blood clotting time which depended principally upon the vitamin K level in the diet.

In order to make certain that the actual consumptions of vitamin K were comparable from group to group, careful records of food consumption and growth were kept. These data may be effectively summarized by the statement that growth and food consumption were not appreciably different within the groups of any one experiment and, in fact, did not vary to any significant extent in the separate experiments. This, of course, is consistent with the fact that vitamin K is not a growth factor.

It was stated in a former paper [Almquist & Stokstad, 1937] that there was no evident relation between vitamin K deficiency and haemoglobin level in non-haemorrhagic individuals. Exception to this statement has been taken by Thayer *et al.* [1937] who have reported restoration of both blood clotting power and haemoglobin level after the administration of vitamin K to deficient chicks, but who did not distinguish in their report between haemorrhagic and non-haemorrhagic individuals. It is well known that haemorrhagic chicks suffer from anaemia induced by loss of blood into the tissues and that within 2 or 3 days after administration of adequate doses of vitamin K the blood may be reabsorbed and the haemoglobin level restored toward normal.

During the course of these and other studies, haemoglobin measurements were made on the blood of individual chicks with prolonged clotting time. Measurements were made in these cases with the New Dare Haemoglobinometer, the mean of 5 or more closely agreeing readings being taken as the final value. Eleven chicks, 2 weeks old, with individual clotting times over 60 min. in each case, were found to have haemoglobin levels ranging from 8·10 to 9·60 g. per 100 ml. with a mean of 8·56. None of these chicks were afflicted with visible haemorrhages. The mean value compares favourably with one of 7·97 obtained by Harmon [1936] with the identical instrument in the case of 2-week old normal chicks fed on a practical ration. Four additional non-haemorrhagic chicks with individual clotting times greater than 60 min. were tested at an age of 3 weeks. The haemoglobin levels found were 7·8, 8·4, 9·0 and 9·7 g. per 100 ml. These values are far above what may be considered an indication of anaemia.

One of the most serious obstacles to accuracy in vitamin K assay by any procedure is the large variability of blood clotting time encountered, although source of chicks, level of vitamin K intake and reserve of the chicks are made as nearly uniform as possible. This variability is not appreciably lowered by previous severe depletion of the chick. It remains high even in chicks that have all received the same amount of the vitamin in the diet for as long as 5 weeks and that have grown equally well.

In a search for other factors influencing the blood clotting time, we measured the clotting times of chicks maintained on the same low dietary vitamin K level, in certain individuals only after complete anaesthesia induced by subcutaneous injection of sodium pentabarbital (Nembutal), and in others in the usual manner. A reduction in blood clotting time due to a condition of fright seemed possible. Such an effect would probably be minimized in birds that had been under the influence of a powerful anaesthetic for ½ hr. or more. However, over a range of 6–19 min., no appreciable differences in average clotting time were found.

Second samples of blood taken immediately after the first samples and from the same incision usually clotted in slightly less time than the first samples. This was also found to be the case for first and second samples taken from different incisions, one on each wing of the chick. In no case was more than approximately 0·2 ml. taken per sample. Since there was no appreciable loss of blood while these samples were being taken, the quantity of blood lost could scarcely have exerted any influence on the blood clotting time. It does not

appear that this reduction in clotting time was due to contamination of the second blood sample by fluids from the cut tissues, since the same slight shortening of the clotting time was observed whether the samples were taken from one cut or from different cuts in the same bird. Furthermore, the incisions were made in such a manner as to avoid cutting of tissue other than the vein.

In a further study of this shortening of the clotting time it was found that the differences in the average clotting times of the first and second blood samples lacked significance in the statistical sense, although there was a consistently shorter average time for the second samples.

None of the above findings aid in explaining the large variability in blood clotting times in chicks of the same group. It is logical to expect that a large part of such variability may be caused by individual differences in the chicks in regard to their vitamin K metabolism. In an attempt to secure a measure of such individual source of variability, we have obtained at intervals of a few days the blood clotting times of 57 identified chicks maintained on the same diet low in vitamin K. Data illustrating the characteristically different blood clotting times of certain chicks reared together upon the same diet and vitamin K level are given in Table IV.

Table IV. *Blood clotting times in minutes of individual chicks fed on the same diet low in vitamin K*

	Age in days			
Chick no.	21	24	27	29
5370	>30	>30	>30	>30
5389	24·4	>30	>30	26·0
5392	>60	>30	21·5	>30
5360	7·0	7·1	4·7	5·4
5378	2·9	5·7	2·8	3·6
5386	13·4	9·7	8·6	10·5

This group of chicks was of random selection. Individuals in the group grew at rates which varied over a twofold range. A small positive correlation between chick weight and blood clotting time was indicated by the coefficient, $0{\cdot}276 \pm 0{\cdot}086$, which is barely significant. This tendency for blood clotting time to be influenced by rate of growth can be almost entirely obliterated by selecting chicks of uniform size at 1 week of age.

Since it has been shown by Greaves & Schmidt [1937] that lack of bile interferes with the absorption of vitamin K in the rat, it seemed possible that chicks in a state of chronic deficiency might be cured by addition of bile acids to their diet, if one cause of their deficient condition were poor absorption of vitamin K. Experiments were conducted using chicks of prolonged blood clotting times; however, the addition of the bile acids, cholic acid, deoxycholic acid, dehydrocholic acid and taurocholic acid to the diet at a level of 0·5% had no detectable effect on the average blood clotting time after 1 week of such feeding, although a uniform, nearly adequate level of vitamin K was present in the diet. Control groups also showed no appreciable or general changes. The characteristic clotting time differences, as illustrated in Table IV, were evidently not due to a lack of bile acids in the digestive tract.

As a result of the large individual variability of chicks in regard to blood clotting time, it becomes evident that a reduction of the probable errors in average blood clotting time requires primarily an increase in the number of chicks per test group. This means that any procedure requiring complicated and lengthy

treatment of each animal and of each blood sample will be extremely laborious as well as more susceptible to experimental errors. For these reasons we have sought to perfect the most simple method of conducting a vitamin K assay.

In the procedure now adopted by us 10 chicks (more when greater accuracy is desired) that have been maintained on the basal diet for 1 week since hatching are placed in each test group and fed on a supplemented basal diet from 7 days to 14 or 21 days. The purposes served by maintaining the chicks for 1 week on the basal ration are many, e.g. depletion of the reserve, detection of weak, defective and slow-growing chicks and elimination from the test pens of most of the usual early chick mortality. Included with the test groups receiving the supplement intimately mixed with the diet are a negative control group having no supplementary vitamin K in the diet and several groups on diets containing various levels of the reference standard.

The reference standard could, of course, be assigned a certain "unit" value, but we have preferred not to follow the common tendency to define "units" and possibly lead vitamin K into the confusion of meanings and definitions that has been troublesome in the case of certain other vitamins. It would seem better to defer such definitions until requirements can be based upon a weight of a pure or standard preparation.

As previously shown, the age of 14 days is one of maximum susceptibility to inadequate vitamin K intake. An assay can therefore be terminated at this time, after 7 days of supplementary feeding, provided that a series of suitable reference standard control groups are available and all chicks are of a common origin and have received the same treatment.

For more precise assay it is preferable to continue the supplementary and control feeding to 3 weeks of age. As indicated in Table III, chicks at this age seem to attain a final adjustment of blood clotting time to the vitamin K level of the diet. The results of Table III have been used in constructing Fig. 1, which expresses the relation of the logarithm of the vitamin K concentration in the diet to the reciprocal of the average blood clotting time. The relation is evidently linear, which means that the clotting power of the blood, as expressed by the reciprocal of the clotting time, is a simple function of the logarithm of the vitamin K concentration in the diet. The line relating the reciprocal of the clotting time to the logarithm of the dietary vitamin K concentration appears to extrapolate toward the origin. This implies that, as the vitamin K level is decreased, blood clotting time tends to become extremely prolonged.

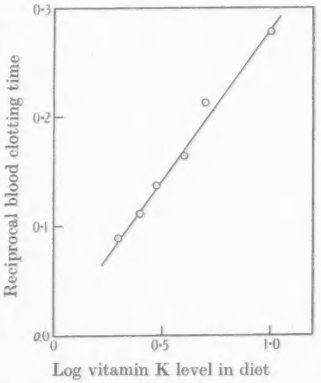

Fig. 1. Relation of the reciprocal blood clotting time to the logarithm of the vitamin K level in the diet (ml. reference standard sol. per kg. diet).

For the curve given it was found that

$$\log \text{vitamin K concentration} = \frac{3{\cdot}6}{\text{blood clotting time}}.$$

It is probable that the constant in this equation will not hold in all cases for chicks of this age and that the interpolation of the results of any assay should be made separately for each series. Although the same equation was obtained from the results at 4 weeks, the constant was definitely lower, approximately 2·8.

This constant would be expected to decrease with increasing age of the chicks.

The linear relation of the logarithm of the dietary vitamin K level to the reciprocal of the blood clotting time affords a very useful means of interpreting average blood clotting times of test groups in terms of the potency of the reference standard. Thus, for interpolating the results of an assay it is necessary to have only two accurately determined points from reference standard groups.

It appears inadvisable at present to specify certain values for blood clotting times to serve as a basis for defining units or activity of vitamin K concentrates, as Thayer *et al.* [1938] have proposed. These workers have defined a unit of vitamin K as that quantity of material required to reduce the clotting time of the blood of 50 % of the chicks to 10 min. or less. A clotting time of 10 min. or less was considered normal. It is only necessary to recall the variability of blood clotting time with age (Tables I and II) to realize that highly erroneous results can be obtained by such method of assay unless the ages of chicks and the vitamin K reserves of chicks are closely standardized.

The fact that on low dietary levels of vitamin K chicks with only a moderate K reserve tend to show at 2 weeks a maximum blood clotting time, which later decreases, while chicks with a greater reserve may not show such marked changes, has several interesting implications. The day-old chick weighs 30–40 g. and has a corresponding, definite and increasing requirement for essential food factors. However, the intake of such factors from the diet starts at zero from which it increases as the chick consumes food. The chick must soon obtain entirely from the diet the essential factors which it may have carried in reserve at the time of hatching. During the first days of the chick's life, therefore, the reserve is being depleted pending the acquisition of a sufficient quantity of essential food factors from the diet. When one of these food factors is limited by a low intake in the diet, the possibility of deficiency is greatly increased and, when the reserve of the chick is also low, a seriously deficient condition is almost certain.

During the period from 1 to 2 weeks the chick makes the maximum gains per unit of food consumed. It is during this period of most rapid gain in relation to food consumed that the chick is most affected by an inadequate dietary supply of vitamin K. Even in Exp. II, where the chicks had a high reserve of vitamin K, it was found that the greatest increase in blood clotting time took place during the second week. It is significant, in this respect, to note that Harmon [1936] has shown that at an age of 2 weeks the haemoglobin levels of normal chicks pass through a minimum value which is elevated again at 3 weeks.

After an age of 2 weeks the gain in wt. per unit of food consumed decreases and it is found that the blood clotting time also drops, indicating that the requirement of vitamin K per unit of diet has decreased. This decrease is probably analogous to the lowering of the vitamin G dietary levels required by chicks [Heuser *et al.*, 1938].

Summary

1. The blood clotting time of the chick varies with the age and the vitamin K reserve in the chick, and the vitamin K level in the ration, and tends to reach a maximum at 2 weeks of age.

2. At 3 weeks of age the chick achieves a balance of blood clotting power with respect to the vitamin K level in the ration.

3. The reciprocal of the blood clotting time is a simple linear function of the logarithm of the vitamin K level in the ration, over a practical range of values.

4. Chicks free from haemorrhage but with greatly prolonged blood clotting times had normal haemoglobin levels.

5. An improved assay procedure for vitamin K is suggested.

The authors wish to acknowledge valuable assistance received from Dr I. M. Lerner in the statistical analysis of data and from the Works Progress Administration (AP No. 465–03–3–209) in the care of animals and in the recording and treatment of data.

REFERENCES

Almquist & Stokstad (1937). *J. Nutrit.* **14**, 235.
Greaves & Schmidt (1937). *Proc. Soc. exp. Biol., N.Y.*, **37**, 43.
Harmon (1936). *Poult. Sci.* **15**, 54.
Heuser, Wilgus & Norris (1938). *Poult. Sci.* **17**, 105.
Thayer, MacCorquodale, McKee & Doisy (1938). *J. biol. Chem.* **123**, cxxi.
—— McKee, MacCorquodale & Doisy (1937). *Proc. Soc. exp. Biol., N.Y.*, **37**, 417.

CCXLV. THE TIME FACTOR IN THE INTERACTION OF AMINO-ACIDS WITH SUGARS

By MAX FRANKEL and AARON KATCHALSKY

From the Hebrew University, Jerusalem

(*Received 8 June 1938*)

In a previous communication we have shown [Frankel & Katchalsky, 1937] that the interaction of α-amino-acids and peptides with sugars in slightly alkaline, neutral and slightly acid solution leads to a decrease in *p*H owing to the disappearance of basic amino-groups, but no quantitative conclusions were drawn. It was mentioned that the interaction in question "proceeds over a relatively long time interval". In the experiments now reported the time progress of the decrease in *p*H is described in greater detail.

The study of the time factor throws light also on a recent paper by Balson & Lawson [1938] who, on the other hand, state that the *p*H decreases shown by amino-acids or peptides in mixture with sugars is solely attributable to the acidic properties of the sugars themselves. The discrepancy between the results of Balson & Lawson and our own is, we believe, due mainly to the fact that, in choosing their experimental technique, these authors failed to take into account the time factor of the interaction. The interaction between the aldoses and amino-acids or peptides becomes noticeable, under certain conditions, only after some time. In the experiments described by us intervals of 20–30 min. were allowed between NaOH additions in the potentiometric technique, and observations in the colorimetric experiments were extended over periods of 24–48 hr. Balson & Lawson, on the other hand, used their special technique [1935] which enables them to shorten even that time interval which is normally required in potentiometric titrations between consecutive additions, and thus created markedly unfavourable conditions for observations of the interaction.

Other minor causes of the discrepancy are dealt with later in this paper.

In order to study the time factor more fully we carried out the present experiments as follows.

I. Mixtures of amino-acid and sugars were titrated by the potentiometric method under different time conditions: in (1) the interval between each addition of NaOH was 10 min., in (2) the interval was 3 hr. Time in each case was counted from the moment at which initial potential was attained.

II. Equal parts of mixtures containing both components of the reaction were brought to desired *p*H values by addition of NaOH and both the initial *p*H and the changes of *p*H were determined electrometrically during a period of several hours.

In all cases controls containing either the amino-acid or the sugar at the *p*H and concentration of the test mixtures, were run.

The amino-acid was glycine. The sugars were: sucrose and fructose for non-aldehydic, non-reactive sugars; glucose and galactose for the aldehydic sugars.

The experiments were carried out under sterile conditions at 20° throughout.

Results

I. *Potentiometric titrations with different time intervals between NaOH additions*

(*a*) *Glycine + non-aldehydic sugars*

1. *Time interval between additions—10 min.*

When the slight correction necessitated by the acidity of the sugars is allowed for (0–3 mV. for sucrose, 0–6 mV. for fructose) the curves obtained are identical with that of glycine + water.

2. *Time interval—3 hr.*

Once the potentials are established no change in *p*H occurs. The titration curves of the solutions are therefore the same whether the NaOH is added at intervals of 10 min. or 3 hr.

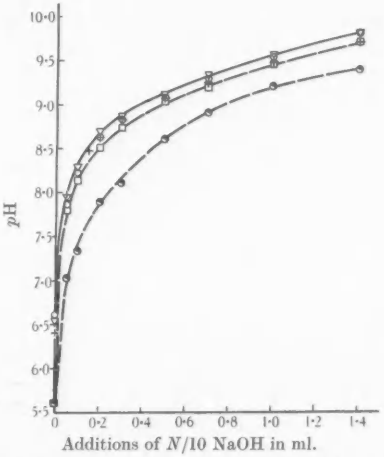

Fig. 1. Titration curves of glycine-sugar mixtures with different time intervals between NaOH additions. ▽ 1 ml. glycine *M*/4 + 1 ml. H_2O. ○ 1 ml. glycine *M*/4 + 1 ml. sucrose *M*/1 (time interval 10 min. or 3 hr.). + 1 ml. glycine *M*/4 + 1 ml. fructose *M*/1 (time interval 10 min. or 3 hr.). □ 1 ml. glycine *M*/4 + 1 ml. glucose *M*/1 (time interval 10 min.). ● 1 ml. glycine *M*/4 + 1 ml. glucose *M*/1 (time interval 3 hr.).

Table I. *Potentiometric titration of 2 ml. of mixtures containing glycine* M/*8 and glucose* M/*2 by NaOH* N/*10, each addition of alkali being made* (*1*) *10 min. or* (*2*) *3 hr. after the initial potential was attained.*

Additions of alkali (ml.)	*p*H (1)	*p*H (2)
0·0	5·61*	5·61*
0·05	7·71	7·03
0·1	8·13	7·34
0·2	8·51	7·89
0·3	8·73	8·11
0·5	9·04	8·61
0·7	9·21	8·91
1·0	9·47	9·21
1·4	9·72	9·41
1·8	9·96	9·90

* The measured *p*H; a decrease in *p*H might have occurred here during the mixing and measuring.

(b) Glycine + aldehydic sugars

1. *Time interval—10 min.*

In this case also the necessary correction for the acidity of the glucose is small (0–4 mV.) within the *p*H range and concentration ratio of the experiments.

Even when the potentiometric titration was conducted at time intervals of 10 min. the potentials initially attained tend to decrease. Yet the decrease under these conditions (Δ *p*H about 0·1), though greater than the range of error, is still rather small (Fig. 1; Table I (1)). Balson & Lawson [1938] correctly concluded that a *p*H lowering of this size is too small for an interaction to be inferred.

2. *Time interval—3 hr.*

The *p*H values found after each addition were lower than the corresponding values obtained with additions at intervals of 10 min. by as much as 0·8 *p*H. (Cf. Fig. 1, Table I (2).)

II. *Electrometric* p*H measurements during 3 hr. in mixture solutions brought by additions of NaOH to different initial* p*H values*

(a) Glycine + non-aldehydic sugars

The sugars were either sucrose or fructose.

All *p*H values remained constant at their initial level for 3 hr. Sterile mixture solutions at *p*H up to 8·8 and sterile acid and neutral controls underwent no change in *p*H within one or more days; both sugar controls and mixtures of higher alkalinity undergo *p*H depressions when kept for 24 hr. or more. This result is to be expected in view of the known instability of sugars in alkaline solution.

(b) Glycine + aldehydic sugars

Glycine + glucose. Marked and rapid decreases in the *p*H values were observed (Table II). The velocity of the depression decreased with increasing alkalinity. All *p*H values attained constancy at least 30 min. before the end of the observation period.

Table II. *Potentiometric measurements of* p*H changes with time in 2 ml. mixtures containing glycine* M/8 *+ glucose* M/2 *+ x ml. NaOH* N/10

Time after mixing min.	x = ml. NaOH $N/10$					
	0·05	0·1	0·3	0·5	1·0	1·8
			*p*H values			
25	7·71	8·13	8·73	9·04	9·43	9·95
30	7·63	8·08	8·71	9·02	9·40	9·93
37	7·47	8·01	8·68	9·01	9·40	9·89
45	7·34	7·94	8·65	8·99	9·40	9·89
60	7·23	7·89	8·64	8·99	9·40	9·89
75	7·17	7·78	8·61	8·95	9·38	9·89
150	7·17	7·47	8·47	8·88	9·36	9·87
180	7·17	7·47	8·46	8·88	9·35	9·87

In sterile mixtures and controls of *p*H < 8·3 additional decreases did not occur even after several days. In the alkaline range, above *p*H 8·3, glucose controls prepared and maintained under sterile conditions showed depressions in *p*H after one or more days showing decomposition of the sugar. The mixtures under the same conditions also showed additional depressions. It is obvious that

sugars which are allowed to stand for a longer period in strongly alkaline media undergo chemical changes which, as in certain cases observed by the authors and others, are aided by the presence of amino-acids. As has previously been shown, more strongly alkaline media induce a new type of reaction in the mixtures. It seems, therefore, advisable to reduce the observation time, which in the colorimetric experiments was extended over one or two days, to an interval of e.g. 3 hr. within which no decomposition of the sugars occurs.

It may be noted here that the observations on the diminution of the *p*H decrease at higher alkalinities were taken at points far enough from the end-point of the titration to be uninfluenced by the final convergence of the titration curves.

Galactose when mixed with glycine behaves in the same way as glucose, except that within a *p*H range of 7–8 the readings with galactose became constant within 20 min., whereas in the *p*H range 8–9 constancy is attained after 2 hr.

The molar concentration ratio of amino-acid to sugar in our previous experiments was 1 : 1, and in the mixtures the concentration of each component was *M*/4. The corrections for the acidity of the sugars in the *p*H range investigated were found to be between 1–2 mV. and were therefore practically negligible.

It may also be remembered that in our previous colorimetric experiments the acidity of the sugars was eliminated by equalizing the *p*H of both component solutions before mixing (principle of isohydric solutions).

Lastly it may be mentioned, in anticipation of a later paper, that leucylglycine, the peptide investigated by Balson & Lawson, is less reactive when mixed with aldoses than glycine or its peptides in which the free NH_2-group belongs to the glycine moiety.

Summary

1. Mixtures of glycine and either non-aldehydic or aldehydic sugars were potentiometrically titrated under different time conditions.

No *p*H depression occurred in mixtures containing non-aldehydic sugars and no influence of the length of the intervals between NaOH additions on the titration curve was detectable. In mixtures containing glucose a *p*H depression appeared which was much more marked when the time interval between the NaOH additions was extended.

2. No change within at least 3 hr. in the *p*H values occurred in mixtures of glycine and non-aldehydic sugars brought to different initial *p*H. Under the same conditions marked and rapid decreases in the *p*H values occurred in mixtures containing glycine and either glucose or galactose.

3. The view of Balson & Lawson, that the *p*H depressions observed by us are to be attributed solely to the acidic properties of the sugars is shown to be incorrect.

4. Owing to different experimental conditions no direct conclusion in regard to our previous findings can be drawn from the results of Balson & Lawson.

REFERENCES

Balson & Lawson (1935). *Biochem. J.* **29**, 2700.
—— —— (1938). *Biochem. J.* **32**, 230.
Frankel & Katchalsky (1937). *Biochem. J.* **31**, 1595.

CCXLVI. THE EFFECTS OF ANAESTHETICS AND OF CONVULSANTS ON THE LACTIC ACID CONTENT OF THE BRAIN

By WILLIAM ELLIS STONE

From the Laboratory of Physiological Chemistry, University of Minnesota, Minneapolis

(*Received 17 August 1938*)

That disturbances in the intermediary carbohydrate metabolism of the brain may be involved in certain "functional" disorders of the central nervous system is indirectly suggested by many observations. The beneficial effect of the "insulin shock" therapy introduced by Sakel [1937] for the treatment of schizophrenia is a particularly striking example, since one of the effects of hypoglycaemia is to diminish the supply to the brain of glucose, the normal substrate for oxidative catabolism in this organ [Dameshek & Myerson, 1935].

In epilepsy, also, the brain metabolism may be abnormal. Some form of anoxaemia has long been considered a possible etiological factor in convulsive disorders [Cobb, 1936]. The seeming antagonism between epilepsy and schizophrenia led Meduna [1937] to introduce the metrazol convulsion therapy for schizophrenia.

An older theory of narcosis, recently given a new aspect by the work of Quastel and his coworkers [Quastel & Wheatley, 1932; 1934; Jowett, 1938], suggests that an interference in brain oxidations may be involved in narcosis. Such an interference may be important in the prolonged narcosis therapy for schizophrenia [Palmer, 1937]. The work of Quastel & Wheatley [1933] also suggests a toxic effect of amines on brain metabolism as of possible importance in mental disorder.

The observations of Loevenhart *et al.* [1929] on the temporary effects of cyanide injection and of inhalation of high carbon dioxide-air mixtures on schizophrenic patients are of interest also.

The mechanism of glucose catabolism in the brain is a very controversial subject at present. Whether lactic acid is a direct intermediate, or whether it arises from pyruvic acid as a side reaction, is not known with certainty. But in either case, a disturbance at any point in the reaction chain or chains involved in glucose catabolism might be expected to cause an increase or decrease in the concentration of lactic acid in the brain. The object of this investigation was to discover whether or not such an increase or decrease is caused by anaesthetics or by convulsant drugs.

With the exception of the work of Kerr and coworkers [1936; 1937; Avery *et al.* 1935], the brain lactic acid values given in the literature are not dependable, because of post-mortem glycolysis. Kerr and coworkers froze the brain *in situ* in the living animal, but their technique required the use of anaesthetics, which decrease the brain lactic acid, as will be shown.

Experimental

Mice were used in this investigation. These animals are small enough to be plunged into liquid air and frozen, without the use of anaesthetics or surgical procedures. The mouse is frozen solid in 2–3 sec., and reaches the temperature

of the liquid air in 60–90 sec. The brain is then removed, using chilled instruments and working on a surface consisting of a strip of cardboard chilled in liquid air. The tissue, instruments, and cardboard are chilled at intervals during the operation, so that the brain does not soften at any time. The whole brain is removed, and stored in liquid air, ready for crushing.

The crusher (Fig. 1) is similar to that described by Graeser *et al.* [1934], but much smaller, and simplified. The crushing surfaces are of tool steel; the ring and base of iron. The crusher is chilled by submerging for a few sec. in liquid air, the frozen tissue then placed on the anvil, with the ring in place, and the tissue crushed by several blows of a hammer on the steel plunger. The crushed tissue forms a solid disk which sticks in the iron ring, and is poked out with a glass rod into the $ZnSO_4$-H_2SO_4 contained in a tared centrifuge tube (with rubber stopper). In order to avoid loss of tissue during the transfer, a glass funnel with the stem cut off is used. The tube is restoppered, shaken well, and weighed.

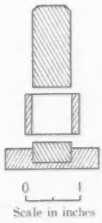

Fig. 1. Tissue crusher.

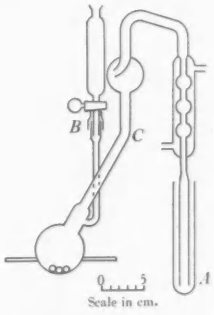

Fig. 2. Distillation apparatus.

Determination of lactic acid

Lactic acid was determined by a modification of the method of Friedemann & Graeser [1933]. Since a mouse brain weighs only 0·3–0·4 g., it was necessary to use much smaller quantities than those employed by Friedemann & Graeser.

The distillation was carried out in the pyrex glass apparatus shown in Fig. 2. All rubber connexions have been eliminated except that at *B*, which does not become heated, and here the rubber does not come into contact with the solution. The apparatus is easily rinsed by inverting and running water in at *A* and out at *B*. Draining through *B* is satisfactory unless the angle at *C* has been made too acute; 150° is about right. The apparatus is easily constructed from a 100 ml. distilling flask and the micro-Kjeldahl distillation apparatus described by Cavett [1931].

Protein removal is accomplished by the $Zn(OH)_2$ method. The crushed tissue is added to 3 ml. solution containing per litre 35·5 g. $ZnSO_4$, $7H_2O$ and 355·5 ml. *N* H_2SO_4 [Blatherwick *et al.*, 1935]. An amount of 0·5 *N* NaOH is added sufficient exactly to neutralize the acid solution (determined by previous titration, using phenolphthalein). Water is then added to make a total volume of 7·5 ml., counting the tissue 80 % water. The mixture is allowed to stand for 30 min. with occasional shaking, and centrifuged.

To 5 ml. supernatant fluid are added 0·6 ml. $CuSO_4$ (containing 200 g. $CuSO_4$, $5H_2O$ per l.), and 0·6 ml. $Ca(OH)_2$ suspension (prepared by slaking 200 g.

fresh lime and diluting to 1 l.). Water is added to the mixture to make a total volume of 15 ml. This is allowed to stand for 30 min. with occasional shaking, centrifuged and decanted.

A 13 ml. aliquot of the solution is introduced into the distillation apparatus (at *B*), followed by 2 ml. of H_3PO_4-$MnSO_4$ mixture (100 g. $MnSO_4$, $4H_2O$ and 25 ml. 85 % H_3PO_4 per l.), and 5 ml. water. The funnel is then connected at *B*, with the stopcock closed, and filled to the 15 ml. mark with a colloidal suspension of MnO_2 (freshly prepared by mixing equal quantities of 0·013 % $KMnO_4$ and 0·03 % $MnSO_4$, $4H_2O$ solutions. Rapid flocculation does not occur unless $MnSO_4$ is slightly in excess). The receiver contains 2 ml. 0·1 *M* $NaHSO_3$.

The solution is brought to boiling, and small portions of MnO_2 are run in at intervals, the whole 15 ml. being added during a 10 min. period. The total distillation period is 15 min., the rate being such as to give a final vol. of 20 ml. in the receiver. At the last, the receiver is lowered and the distillation continued for about 1 min. to rinse the outlet. Bumping is prevented by the presence of three glass beads in the flask. After the distillation, the receiver is cooled in ice water.

The excess $NaHSO_3$ is removed by 0·3 *N* I_2, and the end-point adjusted with 0·005 *N* $Na_2S_2O_3$ and 0·0025 *N* I_2, using 1 ml. 1 % starch indicator. The bound $NaHSO_3$ is then released by adding 5 ml. saturated Na_2HPO_4, and titrated with standard 0·0025 *N* I_2 (containing 10 g. KI per l.). A 2 ml. micro-burette is used, and an air-driven stirrer facilitates the titration.

1 ml. 0·0025 *N* I_2 is equiv. 0·1125 mg. lactic acid.

Purification of the reagents used is necessary. Blank determinations usually required about 0·1 ml. of 0·0025 *N* I_2.

Tests on known lactic acid solutions (prepared from Zn lactate) in amounts covering the range involved in these experiments indicated that the results were accurate to within ±3 mg. per 100 g. of brain tissue. Since this is much smaller than the differences between individual normal mice, the method is sufficiently accurate for the present purpose.

Blank tests on phenobarbital, ether, picrotoxin, metrazol, nicotine and NaCN in amounts likely to be present in experiments reported here, showed that these drugs do not form appreciable amounts of $NaHSO_3$-binding substances.

Brain lactic acid in normal mice

Table I gives the data obtained on normal mice. Brain lactic acid values in mice not exercised before killing ranged from 12 to 25 mg. per 100 g. brain tissue (wet wt.), with a mean of 18·9 mg. per 100 g. Neither body wt. nor sex influenced the results significantly. Most of the mice were not subjected to a period of food deprivation before the experiment; such a period was found to have no influence on the brain lactic acid.

It was found that a period of strenuous exercise increased the brain lactic acid significantly. The mouse was placed in a covered beaker, which was then continuously tilted, shaken and rotated, forcing the mouse to exercise in attempting to right itself and maintain equilibrium. The brain lactic acid in these mice was 25–28 mg. per 100 g., with a mean of 26·8, an average increase of 7·9 mg. per 100 g. due to the exercise.

Since in strenuous exercise lactic acid accumulates in the blood, it was desirable to determine whether the increased brain lactic acid was due to diffusion from the blood into the brain. Three mice were injected with 4–8 mg. lactic acid (as Na salt) in 0·56 *M* solution, by tail vein, and killed 5 min. later. The brain lactic acid values agreed with those of normal resting mice; hence it is

concluded that the extra brain lactic acid arising during exercise has its origin in the brain itself, and is due to increased activity of this organ. These observations are in harmony with those of Dameshek & Myerson [1935], who found no absorption by the brain of lactate from the blood in patients in insulin hypoglycaemia (during which brain lactic acid is low). Contrary observations have been made by others, however [McGinty, 1929; Himwich & Nahum, 1932].

Two experiments showed that adrenaline does not increase the brain lactic acid (Table I). These mice each received 0·06 ml. 1/10,000 adrenaline (0·2 mg. per kg.) subcutaneously. In one, this was divided into two equal doses, separated by a 3 min. interval, and the mouse was frozen 3 min. after the second injection. In the other, the adrenaline was given in one dose, producing marked signs of stimulation, and the mouse was frozen 3 min. after injection. These experiments confirm the findings of Kerr *et al.* [1937].

In the tables, the experiments are numbered in the order in which they were carried out.

Table I. *Brain lactic acid in normal mice*

Exp. no.	Wt. of mouse g.	Sex	Brain lactic acid mg. per 100 g.	Remarks
		Without exercise		
1	25	F	23	—
2	24	F	25	—
10	24	M	16	—
15	20·5	F	23	—
18	28	F	16	—
23	21·5	M	19	—
30	25·5	M	16	14·5 hr. food deprivation
44	30·5	M	19	4 hr. food deprivation
61	20	M	12	3 hr. food deprivation
67	19	M	20	—
79	19·5	F	18	—
87	19·5	F	20	—
		Mean	18·9	
		After exercise		
5	21	M	25	Exercised 3 min.
9	21	M	27	Exercised 5 min.
14	23·5	F	27	Exercised 4 min.
19	32·5	F	27	Exercised 4 min.
27	21	M	28	Exercised 5 min.
		Mean	26·8	
		After Na lactate injection		
77	22	M	24	Injected 4 mg. lactic acid
78	26	F	14	Injected 4 mg. lactic acid
86	20·5	F	17	Injected 8 mg. lactic acid
		Mean	18·3	
		After adrenaline injection		
90	31·5	M	17	2 injections, 3 min. apart
91	26·5	M	16	1 injection

Effects of anaesthetics

Table II gives data obtained on mice killed in a state of anaesthesia. The experiments are arranged in the order of increasing duration of anaesthesia before killing, this period being measured from the time of loss of righting reflexes.

Table II. *Brain lactic acid in anaesthetized mice*

Exp. no.	Wt. of mouse g.	Sex	Induction period min.	Period of anaesthesia min.	Brain lactic acid mg. per 100 g.
		Phenobarbital, excitement stage			
51	23·5	M	7	0	17
58	21·5	M	17	0	8
56	22	M	18	0	8
35	27	F	21	0	12
				Mean	11·2
		Phenobarbital anaesthesia			
41	27	M	15	15	9
49	26·5	M	30	18	8
48	25·5	M	13	22	8
34	24	F	30	42	11
36	18·5	M	30	70	6
50	22·5	M	25	196	14
57	24	M	20	210	6
				Mean	8·9
		Amytal anaesthesia			
47	26	M	43	4*	9
46	29	M	10	15	8
45	23·5	F	10	23	8
43	26·5	F	10	25	7
				Mean	8·0
		Ether anaesthesia			
55	24	M	1	0·5	16
81	23·5	M	1	1	18
76	24·5	F	3	5	8
80	20·5	F	1	10	12
71	23·5	F	3	15	13
72	25·5	M	0·5	15	11
75	23	M	3	20	9

* After a second injection.

The barbiturates were administered intravenously (tail vein), or in some cases subcutaneously, when the needle missed the vein. The manner of administration seemed to make little difference. Phenobarbital dosages were 0·04–0·06 ml. 10% phenobarbital as the Na salt per mouse. Amytal dosages were 0·1–0·15 ml. 5% Na amytal per mouse.

All three anaesthetics decreased the brain lactic acid significantly. With phenobarbital, it was shown that the decrease begins during the excitement stage. With ether, which induces anaesthesia much more quickly, the lactic acid decrease lags behind the onset of anaesthesia.

The lactic acid values obtained are lower than those reported by Kerr and coworkers for dog, cat and rabbit brains. However, it must be remembered that they analysed grey matter only, while the whole brain was used in the experiments reported here.

Effect of insulin

Table III gives the data obtained on mice killed in "insulin shock". The mice were deprived of food for periods of 3–16 hr. before the experiments. No correlation seemed to exist between the length of this period and the severity of symptoms or the brain lactic acid. The insulin used was 40 units per ml., Eli Lilly Company. Injections were into the tail veins, or occasionally subcutaneous.

In all mice killed during insulin convulsions, and in some killed while in a flaccid condition, the brain lactic acid was much lower than normal. This

Table III. *Brain lactic acid during "insulin shock" in mice*

Exp. no.	Wt. of mouse g.	Sex	Insulin injected units	Period of insulin action min.	Brain lactic acid mg. per 100 g.
			Killed while in flaccid condition		
25	25·5	M	1, 1, 2*	268	14
26	22	M	1	107	16
28	23·5	M	1, 1, 1*	240	2
68	20·5	M	2	35	6
			Killed during convulsions		
29	21·5	M	1	63	6
31	28·5	M	3	80	5
37	24·5	M	3	230	5
38	25	F	3	53	9
42	29	M	3	88	9
				Mean	6·8

* At intervals of 1–2 hr. Unsuccessful attempt to produce convulsions.

confirms the findings of Kerr *et al.* [1937], although earlier Kerr & Ghantus [1936] reported normal brain lactic acid values after insulin.

A low level of brain lactic acid during hypoglycaemia is in harmony with the view that the decreased glucose supply to the brain is an important factor in hypoglycaemic shock.

Effects of picrotoxin

Picrotoxin in large enough doses causes severe epileptiform convulsions in mice. In these experiments, the dosage used was 0·04–0·08 ml. per mouse, of a solution containing 3 mg. picrotoxin per ml. (in 9 % alcohol), Eli Lilly Company. The injections were made into the tail veins, or occasionally subcutaneously.

Table IV. *Mouse brain lactic acid in picrotoxin convulsions*

Exp. no.	Wt. of mouse g.	Sex	Duration of convulsions min.	Brain lactic acid mg. per 100 g.	Remarks
			Killed at beginning of convulsions		
6	21·5	M	—	26	—
7	23	M	—	19	—
12	22	M	—	24	—
20	27	F	—	13	—
21	23	F	—	15	—
24	25·5	M	—	27	—
32	30	M	—	18	—
33	25	M	—	12	—
39	20	F	—	18	—
40	24	F	—	13	—
			Mean	18·5	
			Killed during convulsions		
3	31·6	F	3	49	Very severe convulsion
4	18	F	8	36	Series of light convulsions
8	21	M	6	39	Last convulsion severe
11	32	M	2	27	All of brain except cerebellum
				30	Cerebellum only
13	22	F	7	30	Severe convulsion
16	20	M	5	34	—
17	33	F	10	37	Series of convulsions
22	21	M	6	42	Series of light convulsions
			Mean	36·9	

Convulsions invariably began in 5 to 8 min., being ushered in by a characteristic shaky appearance, hunching of the back and fighting movements of the front feet. In one mouse the convulsions terminated fatally after being allowed to continue for about 15 min.

In one group of experiments, the mice were frozen when the convulsion was just beginning, as judged by these characteristic signs, or immediately after it began. The brain lactic acid values were normal in this group, with a mean of 18·5 mg. per 100 g. (Table IV).

In a second group, the convulsions were allowed to continue for 2–10 min., and the mouse was then frozen during a convulsion. A significant increase in brain lactic acid occurred in this group, the mean value being 36·9 mg. per 100 g.

Kerr & Antaki [1937] reported experiments with picrotoxin, in which, however, the convulsion was terminated with an anaesthetic before the brain was frozen *in situ*. Using ether, they found a higher than normal brain lactic acid after picrotoxin, but in the case of amytal, which requires longer for induction of anaesthesia, and is pharmacologically antagonistic to picrotoxin, normal values were obtained.

Effect of metrazol

Metrazol convulsions were induced by injection of 0·05–0·07 ml. per mouse, of an aqueous solution containing 2 % metrazol (cardiazol) and 0·02 % Na_2HPO_4. The convulsant action was quite irregular. Sometimes a violent convulsion began within 2 sec., while in other cases it was delayed as much as 7 min., and frequently two or more injections were required. If the injection was subcutaneous, the onset was slower than if intravenous. The convulsions were always very brief, lasting only a few seconds. They sometimes ended fatally. Occasionally recovery occurred before the mouse could be frozen; in these cases a second convulsion sometimes occurred.

The brain lactic acid values obtained on mice frozen during metrazol convulsions varied over a wide range, from normal to very high values, with a mean of 26·1 mg. per 100 g. (Table V). The occurrence of some very high values is of importance, considering the very brief nature of these convulsions.

Table V. *Mouse brain lactic acid in metrazol convulsions*

Exp. no.	Wt. of mouse g.	Sex	Brain lactic acid mg. per 100 g.
52	26·5	M	33
53	22·5	M	25
54	26	F	29
59	21	M	20
60	20·5	M	20
62	25	M	27
63	26	M	21
64	26	M	39
65	22	M	29
66	19·5	M	18
		Mean	26·1

Kerr & Antaki [1937] found high brain lactic acid values after metrazol convulsions, even though amytal or ether was administered before freezing the brain *in situ*.

Effect of nicotine

Nicotine convulsions were produced by subcutaneous injections of 5–11 mg. nicotine per kg., in 1·5, 2·5, or 5 % solution. As with metrazol, the response was quite variable. In some cases repeated injections were made. When a convulsion

occurred, it began with co-ordinated running movements, 45 sec.–2 min. after the injection. These merged into the convulsion, which was usually very brief. Occasionally a series of light convulsions occurred. Recovery or death followed usually within 3 min., if the convulsion was allowed to continue.

The brain lactic acid values found on mice killed during nicotine convulsions ranged from 14 to 27 mg. per 100 g., with a mean of 23·2 mg. (Table VI). These results are not significantly different from those obtained on normal mice.

Table VI. *Mouse brain lactic acid in nicotine convulsions*

Exp. no.	Wt. of mouse g.	Sex	Brain lactic acid mg. per 100 g.
82	18·5	M	27
83	19·5	F	24
84	19·5	M	26
85	27·5	M	23
88	19	F	25
89	28	F	14
		Mean	23·2

Effect of NaCN

Cyanide convulsions were found to be difficult to produce. The response was quite variable, and frequently the mouse died without having a true convulsion. However, 4 mice were frozen during convulsions, after injection of 7–10 mg. NaCN per kg., by tail vein, in 0·2, 0·3 or 0·4% solution. In some, repeated injections were given, some of which may have been subcutaneous rather than intravenous. Probably all of these mice would have died very quickly if they had not been frozen.

The brain lactic acid values were very high, the mean being 62·8 mg. per 100 g. (Table VII).

Table VII. *Mouse brain lactic acid in cyanide convulsions*

Exp. no.	Wt. of mouse g.	Sex	Brain lactic acid mg. per 100 g.
69	22	F	82
70	24·5	F	56
73	21	M	51
74	22·5	F	62
		Mean	62·8

The data are in harmony with those of McGinty [1929], who found that impairment of brain oxidations by cyanide causes lactate to be given out to the blood by the brain.

Discussion

The low brain lactic acid values found in anaesthetized mice are in accord with the view that the cerebral metabolism is depressed during anaesthesia. Dameshek *et al.* [1934] found that during amytal or ether anaesthesia a decrease occurs in the amounts of glucose and of O_2 removed from the blood by the brain. Quastel and his coworkers, as well as others, have observed that the O_2 consumption of brain tissue *in vitro* in the presence of certain substrates is depressed by anaesthetics [Quastel & Wheatley, 1932; 1934; Jowett, 1938].

However, the results reported here apparently do not support the hypothesis of Quastel, which attributes the anaesthetic action of the drugs to a specific inhibition of some part of an enzyme system involved in the oxidation of lactate or pyruvate. If this hypothesis were correct, one would expect to find an

accumulation of lactate in the brain during anaesthesia. It seems necessary to postulate some other effect of the anaesthetic on the brain tissue, besides the specific inhibition of oxidations observed by Quastel and coworkers. As to whether lactate formation from glucose is specifically inhibited by these anaesthetics, a search of the literature has revealed no data, although Loebel [1925] found that certain urethanes and higher alcohols inhibit respiration of brain tissue more than anaerobic glycolysis, and increase aerobic glycolysis. Inhibition of glycolysis would not explain all the observations of Quastel and coworkers.

The most obvious hypothesis to account for the variations observed in brain lactic acid content would be to suppose that the brain lactic acid content is dependent on the activity of the tissue, lagging somewhat behind the tissue activity, as in muscle. Thus, the brain lactic acid is low during anaesthesia, higher in the normal condition, still higher during exercise, which involves motor activity and highest during convulsions. McClendon [1912] has shown that tissue activity involves an increase in cell membrane permeability, and since intracellular metabolism and permeability are interrelated in some way as yet not understood, it is possible that certain metabolic changes are secondary to changes in membrane permeability. Such a hypothesis might also explain the observations of Quastel and coworkers. Spiegel & Spiegel-Adolph [1936] have shown that anaesthetics decrease the brain cell membrane permeability *in vivo*, confirming inferences drawn from observations on fish eggs by McClendon [1915] and on algae by Osterhout [1916].

The low lactic acid values occurring during insulin convulsions and during the excitement stage of phenobarbital anaesthesia do not accord well with the view that the brain lactic acid content is dependent on the tissue activity. The low values with insulin may be attributed to lack of the substrate, glucose, and in the case of phenobarbital, a "release" theory might be adopted, which would suppose that the higher centres are first anaesthetized, leaving the lower centres active during the excitement stage. The results with nicotine are also out of harmony with this first hypothesis, since the values are generally lower than those observed with metrazol, and none are out of the normal range.

A second hypothesis to account for the high values which occur during picrotoxin, and occasionally during metrazol convulsions, would be to suppose that these substances inhibit some part of an enzyme system involved in the brain oxidations, the accumulation of a measurable amount of extra lactate lagging somewhat behind the beginning of convulsions. In the case of cyanide convulsions, the symptoms are generally attributed to an inhibition of the cytochrome-indophenol oxidase system. Since the lactate accumulation was much greater with cyanide than with picrotoxin or metrazol, while the convulsions occurred with less regularity, it would be necessary to suppose that the inhibition of oxidation occurred at a different point in the oxidative mechanism. This is easily possible in a system so complex. The results with insulin are in harmony with this second hypothesis, since in hypoglycaemia the normal substrate for oxidation is lacking, and consequently the supply of oxidative energy required for the maintainance of homeostasis is deficient. The results with nicotine do not seem to be in accord with this hypothesis. Nicotine is of special interest in that it inhibits lactic dehydrogenase, increases anaerobic glycolysis, and decreases succinate oxidation by brain tissue *in vitro* [Himwich & Fazekas, 1935; Quastel & Wheatley, 1937]. Despite these reports, nicotine did not cause a significant increase of brain lactic acid *in vivo*.

That the extra lactic acid found in the brain during picrotoxin and metrazol convulsions does not come from the blood is shown by the normal or subnormal

values observed in conditions in which high blood lactic acid occurs, namely after lactate or adrenaline injection and during ether anaesthesia or insulin convulsions.

Finally, it is possible that the high brain lactic acid values occurring during picrotoxin or metrazol convulsions may be due to circulatory disturbances, either cerebral or systemic, resulting in cerebral anoxaemia. Cerebral vascular spasm is a possible cause of convulsions in some cases [Cobb, 1936]. In metrazol convulsions, Corrin [1938] observed temporary cardiac arrest in 10–15% of cases.

Summary

1. A technique is described for determining the lactic acid content of the brain of the mouse, using liquid air and avoiding both post-mortem changes and the use of anaesthetics.

2. The lactic acid content of the brain of the normal mouse was found to vary from 12 to 25 mg. per 100 g. tissue, with a mean of 18·9 mg. per 100 g. This is increased to 26·8 mg. per 100 g. by strenuous exercise.

3. Anaesthetics (phenobarbital, amytal, ether) decrease the brain lactic acid content significantly. With phenobarbital the decrease begins during the excitement stage of anaesthesia; with ether it lags behind the induction of deep anaesthesia.

4. During insulin convulsions, the brain lactic acid is significantly decreased. During "insulin shock" without convulsions, a decrease is sometimes observed. This finding is attributed to the decrease in glucose supplied to the brain by the blood.

5. During picrotoxin convulsions which have progressed for 2–10 min., the brain lactic acid is significantly increased and is above the level observed in normal mice after strenuous exercise. At the beginning of the convulsion, no increase occurs above the level observed in normal resting mice.

6. During metrazol convulsions, the brain lactic acid varies from normal to significantly increased values.

7. During nicotine convulsions, no significant increase in brain lactic acid content was observed.

8. During cyanide convulsions, the brain lactic acid shows a large increase.

9. High brain lactic acid values cannot be attributed to diffusion of lactate from the blood into the brain.

10. Possible interpretations of the data, and their significance in relation to theories of anaesthesia and to the causation of convulsions are discussed.

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REFERENCES

Avery, Kerr & Ghantus (1935). *J. biol. Chem.* **110**, 637.

Blatherwick, Bradshaw, Ewing, Larson & Sawyer (1935). *J. biol. Chem.* **111**, 537.

Cavett (1931). *J. Lab. clin. Med.* **17**, 79.

Cobb (1936). *Med. Clin. N. Amer.* **19**, 1583.

Corrin (1938). *Arch. Neurol. Psychiat.* **39**, 864.

Dameshek & Myerson (1935). *Arch. Neurol. Psychiat.* **33**, 1.

—— —— & Loman (1934). *Amer. J. Psychiat.* **91**, 113.

Friedemann & Graeser (1933). *J. biol. Chem.* **100**, 291.

Graeser, Ginsberg & Friedemann (1934). *J. biol. Chem.* **104**, 149.

Himwich & Fazikas (1935). *Amer. J. Physiol.* **113**, 63.
—— & Nahum (1932). *Amer. J. Physiol.* **101**, 446.
Jowett (1938). *J. Physiol.* **92**, 322.
Kerr & Antaki (1937). *J. biol. Chem.* **122**, 49.
—— & Ghantus (1936). *J. biol. Chem.* **116**, 9.
—— Hampel & Ghantus (1937). *J. biol. Chem.* **119**, 405.
Loebel (1925). *Biochem. Z.* **161**, 219.
Loevenhart, Lorenz & Waters (1929). *J. Amer. med. Ass.* **92**, 880.
McClendon (1912). *Amer. J. Physiol.* **29**, 302.
—— (1915). *Amer. J. Physiol.* **38**, 173.
McGinty (1929). *Amer. J. Physiol.* **88**, 312.
Meduna (1937). Die Konvulsiontherapie der Schizophrenia. (Halle a.S.)
Osterhout (1916). *Bot. Gaz.* **61**, 148.
Palmer (1937). *J. ment. Sci.* **83**, 636.
Quastel & Wheatley (1932). *Proc. roy. Soc.* B, **112**, 60.
—— —— (1933). *Biochem. J.* **27**, 1609.
—— —— (1934). *Biochem. J.* **28**, 1521.
—— —— (1937). *J. biol. Chem.* **119**, 80*P*.
Sakel (1937). *Amer. J. Psychiat.* **94**, 111.
Spiegel & Spiegel-Adolph (1936). *Proc. Soc. exp. Biol., N.Y.*, **34**, 799.

CCXLVII. ENZYMIC PROTEOLYSIS
II. THE FORMATION OF FREE TYROSINE IN PEPTIC DIGESTION

By M. DAMODARAN and P. S. KRISHNAN

From the University Biochemical Laboratory, Chepauk, Madras

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The liberation of free amino-acids during the digestion of proteins by pepsin has recently become the subject of controversy [cf. Abderhalden, 1937]. The view generally held has been that the action of pepsin ends with the fission of proteins into high-molecular complexes from which amino-acids are set free only by the further action of enzymes with specific peptidase function. In considering the evidence for this view it is necessary to bear in mind that most of the experiments on which it is based were carried out more than 30 years ago [Abderhalden, Baumann, Kautzsch, Körösy & London, 1905–7] and were designed mainly to throw light on the physiological question of the fate of protein in the alimentary tract. The procedure adopted in the well-known experiments of Abderhalden, London and co-workers cited above was to analyse the intestinal contents of dogs collected soon after a protein meal either by killing the animals or by means of fistulae established at different points of the alimentary tract. The experiments led to valuable results on which much of our present knowledge of digestion and absorption of proteins is based, but the chemical technique available was hardly adequate to elucidate the finer chemical differences in the mode of action of the proteolytic enzymes concerned. Thus in one paper by Abderhalden *et al.* [1906] we have the interesting statement, "Eine quantitative Methode zur Bestimmung von Aminosäuren aus Gemischen kennen wir nicht". Distillation after esterification was the method adopted for the detection of amino-acids in the mixtures they were dealing with. From stomach brei, in contrast to material obtained from the duodenum, jejunum etc., amino-acid esters were obtained either not at all, or only in traces. In view of the fact that esterification itself involved processes that might cause protein hydrolysis, it is not surprising that Abderhalden [1931] came to the conclusion that peptic digestion differs from that by trypsin and erepsin in not leading to the formation of free amino-acids.

More recently the same conclusion has been reached by Waldschmidt-Leitz & Künstner [1927] on less justifiable grounds. These authors used alcoholic titration for studying the extent of proteolysis of thymus histone and, on the assumption that peptides in contradistinction to amino-acids titrate quantitatively in 50% alcohol (for which we have found no theoretical or practical justification [cf. Harris, 1929]), claim to have proved the absence of amino-acids from peptic digests.

The evidence for the contrary view rests upon the findings of Northrop [1930] and Calvery & Schock [1936]. The earlier claim of Felix [1925] to have isolated lysine from peptic digests of thymus histone (as benzoyllysine) cannot be accepted without further confirmation, for, according to this author the liberation of free lysine was unaccompanied by any increase in amino or carboxyl groups and involves the assumption that the combination of lysine in histone is different

from that in other proteins. Northrop [1930] found that during the auto-digestion of crystalline pepsin "a large amount of tyrosine crystallised out". From 100 g. of egg albumin digested with 20 g. of pepsin for 26 days Calvery & Schock [1936] isolated 565 mg. of tyrosine; from the same amount of pepsin allowed to digest itself only 150 mg. of tyrosine were obtained.

Abderhalden [1937], on repeating these experiments with casein, was unable to obtain any tyrosine by direct crystallization and ascribes Calvery's findings to bacterial contamination, which according to the former is possible even in the strongly acid medium required for peptic digestion. Exception has been taken to these results also on the ground that the tyrosine present might have been formed through the agency of the acid present in the digest [Oppenheimer, 1936].

We have frequently noticed the crystallization of small quantities of tyrosine in concentrating large scale peptic digests of proteins prepared for various researches. But at the same time the validity of the objections raised by Abderhalden and Oppenheimer could not be denied as the digests were usually allowed to stand for varying periods and no special precautions against bacterial contamination had been taken. Calvery also allowed a digestion period of 26 days and the solution from which he obtained his tyrosine had been kept for 4 months in the ice chest. Digestion experiments were therefore carried out with casein, taking all precautions for excluding bacterial action, with proper controls to take into account the possible formation of tyrosine by the action of the acid medium or by the self-digestion of the enzyme employed, and limiting enzyme action to a short period. From a number of such digests, after preliminary treatment with phosphotungstic acid, we have been able to crystallize out appreciable quantities of tyrosine without any difficulty.

Quantitative determinations were also made during the course of digestion of the extent of peptide groups hydrolysed by formaldehyde titration and of the amount of tyrosine not precipitable by phosphotungstic acid by the colorimetric method of Folin & Ciocalteu [1927]. It is perhaps necessary to make it clear that the latter method does not determine free as distinct from combined tyrosine. Being based upon Millon's reaction which requires only the phenolic hydroxyl of tyrosine to be uncombined [Vaubel, 1900] the determination is valid for tyrosine in solution whether free or in peptide combination, though the fact that it is generally applied to hydrolysed protein has probably caused some misunderstanding. In a method due to Zuwerkalow [1927], of which the Folin-Ciocalteu method is a modification, the determination is made on the intact protein and we have found that satisfactory values of the tyrosine content of proteins can be obtained by this procedure. Emphasis has been laid on this point because in similar experiments on peptic digests analytical values meant to show that arginine was present in the form of low-molecular compounds not precipitable by salicylsulphonic acid [Lieben & Lieber, 1934] have been misconstrued [Calvery *et al.*, 1936] to mean the presence of free arginine.

In the present experiments the tyrosine values were determined after different periods of digestion on the filtrates after treatment of the digest with phosphotungstic acid in H_2SO_4; these values, therefore, represent the amount of tyrosine not combined in the form of large polypeptides (which can justifiably be assumed to be precipitable by phosphotungstic acid) and serve the purpose of setting the upper limit to the amount of free tyrosine present at any stage of digestion. The values given in Table I show the progress of peptide hydrolysis and the liberation of tyrosine, in so far as the latter is indicated by the amount present in the phosphotungstic acid filtrate, in a typical experiment. At the

end of 7 days' digestion with pepsin 6·13% of the tyrosine in casein was in a form not precipitable by phosphotungstic acid. Analysis of the digest at this stage also showed that the filtrate from phosphotungstic acid contained 6·02% of the total N of the digest, of which 45% was in peptide form (Table II).

The yield of pure tyrosine isolated from one such digest was 240 mg. from 203·3 g. of casein, equivalent to 1·8% of the total tyrosine present in the casein or 61% of the tyrosine not precipitable by phosphotungstic acid. From the mother liquor from the first crop of tyrosine a second solid fraction weighing 342 mg. could be obtained by further evaporation which, according to colorimetric estimation, contained 103 mg. of tyrosine; but the pure amino-acid could not be isolated from this fraction by recrystallization and it is not possible to say if this tyrosine was present in the free or combined state. In a control experiment without the addition of pepsin, in which an equal weight of casein was kept under otherwise identical conditions of temperature and acidity for a period of 14 days, not only could no tyrosine be isolated but the phosphotungstic acid filtrate did not show the slightest trace of tyrosine free or combined on being concentrated and treated with Millon's reagent. In an enzyme control in which an equal weight of pepsin was allowed to digest itself the phosphotungstic acid filtrate contained less than 17 mg. of tyrosine.

Abderhalden, in contradicting the findings of Calvery, has laid emphasis on the possibility of micro-organisms being active in peptic digests in spite of the acidity of the medium, as lumps of protein are usually present during the initial stages and the interiors of these would not be accessible to the bactericidal action of the acid. In one experiment, therefore, casein suspended in water was first sterilized by heat before the addition of H_2SO_4 and pepsin. From 202·5 g. of sterilized casein digested for 14 days 234 mg. of pure tyrosine and a further 145 mg. of the nearly pure amino-acid were isolated. From sterilized casein without the addition of pepsin, but kept under otherwise identical conditions, no tyrosine could be detected in solution.

There can be no doubt, therefore, that peptic digestion gives rise to free tyrosine. The amount of free amino-acids produced by the action of pepsin may be too small to be of physiological significance on account of the short time during which the chyme remains in the stomach, but in studying the chemical mode of action of the enzyme and its relation to protein structure, the ability of pepsin to liberate amino-acids will undoubtedly have to be taken into account.

Experimental

Rate of liberation of tyrosine

50 g. casein were digested with 2·5 g. pepsin under conditions described later in the section on isolation. Peptide hydrolysis was determined daily on 5 ml. aliquots by formaldehyde titration. Simultaneously, 100 ml. of the digest were pipetted out for determination of tyrosine not precipitable by phosphotungstic acid by the following procedure: the solution was made 5% acidic with 50% H_2SO_4 and treated with a 30% solution of phosphotungstic acid in 5% H_2SO_4 added in slight excess. The precipitate was separated by centrifuging and washed thoroughly with dilute phosphotungstic acid-H_2SO_4. The centrifugate and washings were combined, freed from phosphotungstic acid and H_2SO_4 by baryta which was itself quantitatively eliminated by H_2SO_4, concentrated *in vacuo* at 40° and finally made up to 25 ml. Tyrosine was estimated on 10 ml. aliquots according to Folin & Ciocalteu [1927]. Total tyrosine in the digest was determined by the method of Zuwerkalow [1927].

The extent of peptide hydrolysis at each stage was calculated from the increase in amino-N as determined by formaldehyde titration, by comparison with the amino-N on complete hydrolysis. The value of the latter was taken to be 68·14% of the total N as calculated from Van Slyke's data for the N distribution of casein [Plimmer,1917] by adding together the whole of the amino-N, $\frac{1}{4}$ of the arginine-N, $\frac{1}{3}$ of the histidine-N and the whole of the lysine- and cystine-N; the total N in the solution was estimated in a 5 ml. aliquot by the micro-Kjeldahl method.

The results are summarized in Table I.

Table I

50 g. B.D.H. "Light White" casein + 2·5 g. B.D.H. pepsin in 1 litre approximately $N/20$ H_2SO_4, *p*H 1·8.

Total N = 30·07 mg./5 ml.
Amino-N on complete hydrolysis (calc.) = 20·48 mg./5 ml.
= 14·63 ml. $N/10$ NaOH
Total tyrosine = 261 mg./100 ml.

Days	Formaldehyde titre; ml. $N/10$ NaOH	Peptide hydrolysis %	Tyrosine in phosphotungstic acid filtrate: mg./100 ml. of digest	Tyrosine in phosphotungstic acid filtrate: % of total tyrosine
0	0·83	—	—	—
1	1·97	7·79	4·41	1·69
2	2·43	10·93	6·37	2·44
3	3·12	15·65	7·93	3·04
4	3·44	17·83	12·00	4·60
5	3·53	18·45	13·22	5·07
6	3·62	19·06	15·73	6·03
7	3·70	19·62	16·00	6·13

Composition of the phosphotungstic acid filtrate at the end of digestion

On the 7th day 200 ml. of the digest were pipetted out and treated with phosphotungstic acid as already described. After removal of reagents the solution was concentrated *in vacuo* and made up to 25 ml. Total N (micro-Kjeldahl) and amino-N (Van Slyke) were determined on aliquots. 10 ml. of the solution were hydrolysed with 30 ml. of conc. HCl for 10 hr.; the HCl was removed as completely as possible by distillation to dryness *in vacuo*, the solution made faintly alkaline by adding a 10% suspension of baryta and distilled again under reduced pressure to expel any ammonia present. The residual solution was made up to 25 ml. and amino-N estimated in aliquots. The results are given in Table II.

Table II

	mg./200 ml. digest	%
Total N in digest	1202·6	—
Total N in phosphotungstic acid filtrate	72·4	6·02
Amino-N in phosphotungstic acid filtrate before hydrolysis	37·68	52·05
Amino-N in phosphotungstic acid filtrate after hydrolysis	70·55	97·45
Peptide-N	—	45·40

Isolation of tyrosine

250 g. of B.D.H. light white casein were ground up to a uniform suspension with 2·5 l. of $N/10$ H_2SO_4 at 37° and mixed with an equal volume of sterile water in a sterilized pyrex flask. After adjusting to *p*H 1·8, 7·5 g. of B.D.H. pepsin dissolved in a small quantity of $N/20$ H_2SO_4 were added and the mixture incubated

at 37°. Care was taken to ensure that there were no particles of protein adhering to the sides of the flask above the liquid level and sufficient toluene was added to form a thick layer reaching to the neck of the flask. After a day's digestion the *p*H, which had changed to 2·2, was readjusted and a further 5 g. of pepsin dissolved in 25 ml. of *N*/20 H_2SO_4 added. The *p*H showed no appreciable change after the third day. After the first few days the "paranuclein" separating out of the solution settled to the bottom, giving a clear golden brown supernatant liquid.

After 7 days, when formaldehyde titration showed a hydrolysis of 18·01 %, 3800 ml. of the digest (containing on the basis of N determinations 203·3 g. of casein) were filtered, the clear solution cooled in ice and treated with sufficient 50% H_2SO_4 also previously cooled in ice, to make the whole solution 5% acidic with respect to H_2SO_4. A concentrated solution of phosphotungstic acid in 5% H_2SO_4 was now added with vigorous mechanical stirring until precipitation was complete, the temperature being maintained throughout at 0°. About 600 g. phosphotungstic acid were required. The mixture was left in the ice chest for 2 days after which the bulky precipitate was removed by centrifuging and washed with ice-cold dilute phosphotungstic acid. To the combined solution and washings baryta was added in the form of a thin cream. The precipitate of barium phosphotungstate and sulphate was separated on the centrifuge and washed thoroughly with baryta water. The combined supernatant liquids were then freed from Ba quantitatively by means of H_2SO_4. The final solution, which was about 5 l. in volume and quite colourless, was concentrated under reduced pressure at 45° to about 700 ml. The solution, which at this stage had a pale bluish tinge, was again made strongly alkaline with baryta to precipitate residual phosphotungstic acid, the small precipitate formed centrifuged off and the Ba again quantitatively removed from the solution by dilute H_2SO_4. The solution was again concentrated *in vacuo* to about 125 ml. and then transferred to a crystallizing dish in a vacuum desiccator over H_2SO_4. Characteristic crystals of tyrosine separated overnight. They were filtered with suction, washed with a little ice-cold water, then with alcohol and ether and finally dried in the vacuum desiccator over H_2SO_4. The solid was perfectly white and weighed 243 mg.

A specimen dried *in vacuo* over P_2O_5 at 100° gave on analysis N, 7·597%, amino-N, 7·602 %. $C_9H_{11}NO_3$ requires N, 7·735%. After a single recrystallization from hot water, without the use of charcoal, the values were N, 7·716 %, amino-N, 7·732 %.

The mother liquor on further concentration in the vacuum desiccator gave a thick deposit of solids which was filtered on a glass filter with suction. The material was inclined to be sticky but was obtained as a granular powder by repeated washing with 95% alcohol. It weighed 342 mg. and contained 9·605 % N and 9·171% amino-N. Pure tyrosine could not be isolated from this fraction by recrystallization, but colorimetric estimation showed that it contained 103 mg. of the amino-acid. A trace of the solid gave a violet coloration with quinone in the cold in the presence of a drop of sodium carbonate.

The alcoholic washings from the second crop of solids on evaporation to dryness gave a thick syrup which contained 26·05 mg. total N and 13·02 mg. amino-N indicating the presence of peptides. Tests for tryptophan were negative; Millon's reagent gave a feeble colour.

Substrate control

250 g. of casein were suspended in 5 l. of $N/20$ H_2SO_4 and, after adjustment of the *p*H to 1·8, left in the incubator under toluene. After 14 days the clear liquid was filtered from the unaltered casein and treated with phosphotungstic acid as already described. The slight precipitate that was obtained was filtered off and the filtrate freed from reagents. The final solution, having a volume of about 5 l., gave no colour with Millon's reagent. It was concentrated under reduced pressure to about 500 ml. At this stage also Millon's reaction was negative. The last traces of H_2SO_4 were now quantitatively removed and the solution concentrated to about 10 ml. when a minute amount of crystalline material separated out, the Millon's reaction being, however, still negative. The separated crystals were inorganic.

Enzyme control

2·5 g. of pepsin were dissolved in 1 l. of $N/20$ H_2SO_4 (same concentration of enzyme as used in the digests) and incubated at 37° under toluene. After 7 days 200 ml. of the digest were pipetted out and precipitated with phosphotungstic acid in the usual way. The filtrate, after removal of phosphotungstic acid, etc., was concentrated *in vacuo* and made up to 25 ml. Aliquots of 10 ml. were taken for the duplicate estimation of tyrosine by Folin & Ciocalteu's method. Since the amount of tyrosine contained in the solution was small a known volume of standard tyrosine solution was added to the unknown before making colour comparison and the amount originally present obtained by difference. 200 ml. of the enzyme solution were found to contain 0·692 mg. of tyrosine not precipitable by phosphotungstic acid.

Isolation of tyrosine from sterilized casein digest

Casein prepared according to Cohn & Hendry [1930] was used directly after the final precipitation and washing without drying. Sufficient wet casein to give about 250 g. dry protein was suspended in 2·5 l. of water and sterilized by heating in a steamer for 1 hr. each day on three successive days. To the suspension cooled to 37° 2·5 l. of approximately $N/10$ H_2SO_4 were added slowly and the *p*H adjusted to 1·8. With vigorous mechanical stirring during the addition of acid formation of lumps could be completely avoided. 12·5 g. of pepsin in dilute H_2SO_4 were added and the mixture incubated under a thick layer of toluene. To avoid all chances of bacterial contamination no samples were withdrawn until the end of the digestion period of 14 days. The procedure after this stage was exactly as described before.

From 4·5 l. of the digest which, according to analysis, contained 1·32 g. of tyrosine on the whole and 517 mg. in a form not precipitable by phosphotungstic acid, two crops of crystals weighing 234 mg. and 145 mg. respectively were isolated. The first fraction gave N, 7·36% and amino-N, 7·49% on isolation and 7·67% and 7·65% respectively after one recrystallization. The second fraction which was not recrystallized gave N, 7·85% and amino-N, 7·97 %. The result with sterilized casein suspended in acid without pepsin was exactly the same as with the substrate control already described.

SUMMARY

The liberation of tyrosine during peptic digestion of casein has been quantitatively followed. It is shown that after 7 days' digestion about 6% of the total tyrosine and roughly the same proportion of the total N of the protein are in a form not precipitable by phosphotungstic acid.

From peptic digests of casein, carried out under conditions where bacterial contamination was completely excluded, tyrosine has been isolated in yields amounting to 1·7–1·8% of the total tyrosine in the protein.

The tyrosine produced by self-digestion of the enzyme forms but a small fraction of that obtained from the casein digest.

Action of acid in the concentration necessary for peptic digestion does not lead to the liberation of the free amino-acid from casein.

REFERENCES

Abderhalden (1905). *Hoppe-Seyl. Z.* **44**, 17.
—— (1931). Lehrbuch der physiologischen Chemie, 6th ed., p. 329. Berlin.
—— (1937). *Fermentforschung*, **15**, 315.
—— Baumann & London (1907). *Hoppe-Seyl. Z.* **51**, 384.
—— Kautzsch & London (1906). *Hoppe-Seyl. Z.* **48**, 549.
—— Körösy & London (1907). *Hoppe-Seyl. Z.* **53**, 148.
Calvery & Schock (1936). *J. biol. Chem.* **113**, 15.
—— Block & Schock (1936). *J. biol. Chem.* **113**, 21.
Cohn & Hendry (1930). *Organic Syntheses*, **10**, 16.
Felix (1925). *Hoppe-Seyl. Z.* **146**, 103.
Folin & Ciocalteu (1927). *J. biol. Chem.* **73**, 627.
Harris (1929). *J. biol. Chem.* **84**, 302.
Lieben & Lieber (1934). *Biochem. Z.* **275**, 38.
Northrop (1930). *J. gen. Physiol.* **13**, 739.
Oppenheimer (1936). Die Fermente und ihre Wirkungen, 5th ed. suppl. pp. 672, 845. The Hague.
Plimmer (1917). The Chemical Constitution of the Proteins, Part I, p. 132.
Vaubel (1900). *Z. angew. Chem.* 1126.
Waldschmidt-Leitz & Künstner (1927). *Hoppe-Seyl. Z.* **171**, 290.
Zuwerkalow (1927). *Hoppe-Seyl. Z.* **163**, 185.

CCXLVIII. THE NON-SPECIFICITY OF THE ASCORBIC ACID OXIDASE

By GEORGE ALAN SNOW and SYLVESTER SOLOMON ZILVA[1]

From the Division of Nutrition, Lister Institute, London

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The observation that the retention of *l*-ascorbic acid and its analogues in the body of the guinea-pig was controlled by their stereochemical structure as well as by their antiscorbutic potencies [Zilva, 1935, 1] suggested that the extension of this work to the investigation of the bearing of the spatial arrangement of the molecule on other biochemical reactions might be advisable. It was considered that the advance of the general problem of the mechanism of the biological action of *l*-ascorbic acid in the animal organism might be furthered by this means.

With this end in view attention was directed to the reversible oxidation of *l*-ascorbic acid and its related compounds by enzymes. In a series of investigations the following results emerged. The enzyme first described by Szent-Györgyi [1930; 1931], which oxidizes *in vitro* *l*-ascorbic acid but not phenols, also oxidized the analogues of ascorbic acid. In addition these compounds could be oxidized by the phenolases present in the apple or potato but only in the presence of mono- or polyhydric phenols or the juice of the plants [Zilva, 1934; 1935, 2; Johnson & Zilva, 1937, 1]. From this it was inferred that the dehydrogenation was brought about by quinones formed by the action of the phenolases on the above substrates. Keilin & Mann [1938] have since found that this also holds true of the polyphenol oxidase present in the cultivated mushroom. This enzyme system which is evidently distinct from the ascorbic acid oxidase apparently functions also *in vivo*, since it has been observed that the equilibrium between *l*-ascorbic acid and dehydroascorbic acid in the living tissue of the apple changes progressively with the development of the fruit [Zilva *et al.* 1935; 1938]. Although the ascorbic acid oxidase oxidized all the analogues of *l*-ascorbic acid investigated, the rate of oxidation was markedly influenced by the stereochemical structure of these compounds. In fact a certain parallelism existed between the influence of structure on their rate of oxidation on the one hand and their capacity of being retained in the animal organism on the other [Johnson & Zilva, 1937, 2]. Thus the compounds in which the oxygen ring engages the hydroxyl group to the right of the carbon chain and which invariably possess antiscorbutic potency were oxidized at a much higher rate than their antiscorbutically inactive enantiomorphs. In contradistinction to this the asymmetry of carbon 5 or the presence of a seventh carbon atom in the chain which conditions the intensity of the antiscorbutic activity of the compounds with the ring to the left of the carbon chain had no influence upon the kinetics of the enzymic oxidation.

It remained to ascertain whether the activity of the ascorbic acid oxidase was restricted to ascorbic acid and its analogues or whether it extended to other compounds capable of reducing indophenol. Preliminary experiments with reductone and dihydroxymaleic acid suggested that these substrates may also be oxidized by it [Johnson & Zilva, 1937, 1]. These results, however, were not

[1] Member of the Scientific Staff, Medical Research Council.

sufficiently clear-cut to justify a final conclusion. Having overcome the technical difficulties first encountered it was possible to investigate the problem of the specificity of the enzyme in greater detail. The results obtained with substrates of different chemical constitution form the subject of this investigation.

Technique

The oxidation of the compounds was assessed by means of a Barcroft-Warburg respirometer and the general technique was similar to that already described [Johnson & Zilva, 1937, 2]. The experiments were carried out at 26° and at *p*H 6·0. The *p*H at the end of the experiment never varied by more than 0·2. The volume of the oxygen was calculated for 760 mm. pressure. As a source of enzyme cucumber juice was employed, prepared by freezing the entire cucumber at −20° and pressing out the thawed pericarp. Some extraneous matter was then removed by adjusting the *p*H of the juice to 7·6–8·0, filtering and acidifying to its original *p*H (approximately 6·0). This enzyme preparation was dialysed in cellophane bags at 1° for 2 days against running distilled water. The quantity of enzyme which absorbed 10 μl. of O_2/min. in oxidizing 2·5 ml. 0·01 *N* *l*-ascorbic acid at 760 mm. pressure, 26° and *p*H 6·0 (*M*/15 phosphate buffer) was arbitrarily adopted to represent 10 units. The enzyme was always diluted to 1 ml. and the substrate in the buffer to 1·5 ml. Control experiments were performed in every case.

Results

Reductic acid as substrate

The reductic acid [Reichstein & Oppenauer, 1933; 1934] was a sublimed and recrystallized sample kindly placed at our disposal by Prof. T. Reichstein. It was found by us to be 99–100% pure by indophenol titration.

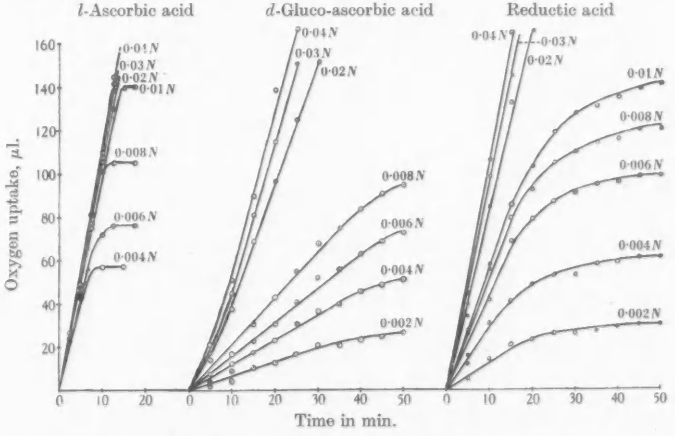


Fig. 1. Rates of oxygen uptake by *l*-ascorbic acid, *d*-gluco-ascorbic acid and reductic acid at varying concentrations expressed in normality. Strength of enzyme (undialysed), 10 units.

The actions of dialysed and undialysed enzyme were studied on this substrate. Since, apart from a difference in the relative rates, the oxidations proceeded similarly, the results obtained with undialysed juice only will be given. Fig. 1

represents the course of oxidation of varying concentrations of *l*-ascorbic acid, *d*-gluco-ascorbic acid and reductic acid in the presence of 10 units of the enzyme. It will be seen that the rate of oxidation of reductic acid, like that of *d*-gluco-ascorbic acid, fell off after a time and was not linear as was found with *l*-ascorbic acid. This falling off was even more noticeable when dialysed juice was used, especially at high substrate concentrations.

In Fig. 2 the oxygen absorbed per min. during the initial stages, represented by linear portions of the graphs in Fig. 1, was plotted against concentrations of substrate. From this it may be observed that for low concentrations an increase of substrate raised the rate of oxidation of *l*-ascorbic acid much more than that of the other two compounds. With higher concentrations, however, such increase had hardly any perceptible effect on the oxidation velocity in the case of the former, but a considerable, although reduced, effect on that of the latter. This phenomenon may possibly be due to a lower combining affinity of the enzyme for *d*-gluco-ascorbic acid and reductic acid.

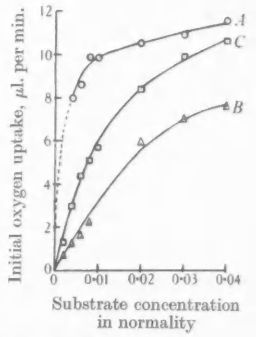


Fig. 2. Initial rates of oxygen uptake by *l*-ascorbic acid, *d*-gluco-ascorbic acid and reductic acid plotted against substrate concentration. Derived from Fig. 1. ⊙—⊙ (*A*) *l*-ascorbic acid. △—△ (*B*) *d*-gluco-ascorbic acid. ⊡—⊡ (*C*) reductic acid.

The results obtained with varying quantities of enzyme and constant concentrations of substrate (0·01 *N*) are given in Figs. 3 and 4. As was to be expected the rate of oxidation was found to be almost directly proportional to the enzyme activity. From Fig. 4 the higher oxidation velocity for *l*-ascorbic acid emerges once more.

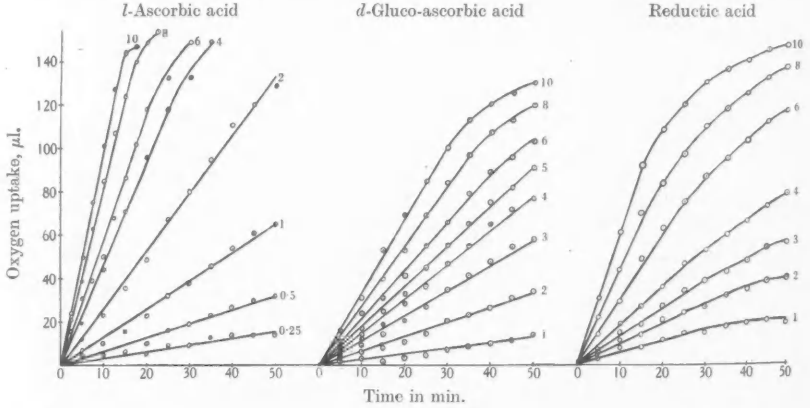


Fig. 3. Rates of oxygen uptake by *l*-ascorbic acid, *d*-gluco-ascorbic acid and reductic acid at varying enzyme concentrations expressed in units. Substrate concentrations, 0·01 *N*. Undialysed juice used throughout.

In order to ascertain whether the amount of oxygen absorbed was equivalent to the amount of substrate destroyed, the following experiment was devised. Out of a battery of eight Warburg flasks treated in the usual manner, each containing the same amount of enzyme and substrate, one was reserved for

manometric readings; the remaining seven were removed singly at suitable intervals and immediately titrated with $N/1000$ indophenol. The results were calculated on the assumption that 1 mol. of each of the substrates required 1 atom of oxygen and the values obtained were corrected to 760 mm. pressure

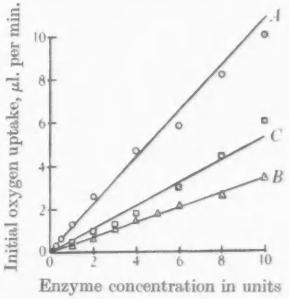


Fig. 4. Initial rates of oxygen uptake by *l*-ascorbic acid, *d*-gluco-ascorbic acid and reductic acid plotted against enzyme concentration. Derived from Fig. 3. ⊙—⊙ (*A*) *l*-ascorbic acid. △—△ (*B*) *d*-gluco-ascorbic acid. ⊡—⊡ (*C*) reductic acid.

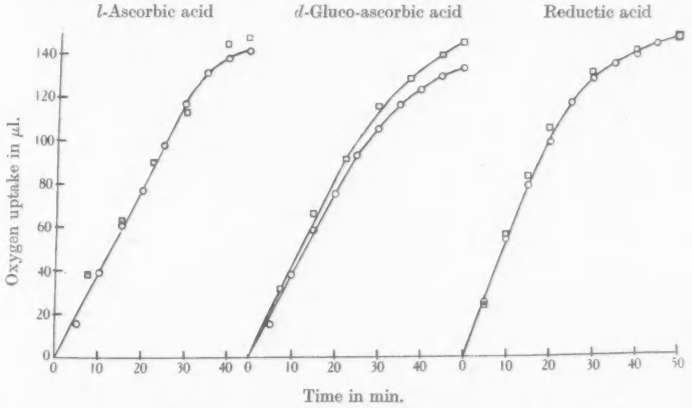


Fig. 5. Comparison of observed oxygen uptakes by *l*-ascorbic acid, *d*-gluco-ascorbic acid and reductic acid in the presence of undialysed cucumber juice, with that calculated from the disappearance of the substrates. ⊙—⊙ observed values. ⊡—⊡ calculated values.

and 26°. It will be seen from Fig. 5 that these figures agree well with those observed simultaneously by direct manometric measurements. We do not consider the slight deviation between the *d*-gluco-ascorbic acid graphs to be significant.

Reductone as substrate

Reductone was prepared by the method of Euler & Klussmann [1933] and Euler & Martius [1933, 1, 2, 3, 4]. The crude material was purified from hot ethyl acetate and then crystallized from butyl alcohol. The slightly brown product obtained in this way did not give a distinct M.P. (charring above 200°) thus resembling the product obtained by Euler & Martius. Further purification

revealed that this material contains a hitherto unsuspected impurity although by indophenol titration it was found to be 97% pure. All the experiments described below were therefore carried out with a preparation which was further purified by sublimation under low pressure at 125°. Two such treatments removed all the non-volatile impurities which remained as a feathery brown residue present to the extent of 8–10% of the crystallized product. The sublimed reductone, colourless needles, melted at 154–156° (decomp. uncorr.) according to the rate of heating. It must be pointed out that anomalous results were obtained when the non-sublimed substance was used as substrate.

Reductone reduces indophenol much more slowly than ascorbic acid and its analogues. It is, however, possible to obtain a definite end point if the titration is carried out at *p*H 4·5 and the indicator added slowly. Sublimed reductone was found to be 98–99% pure by this titration method.

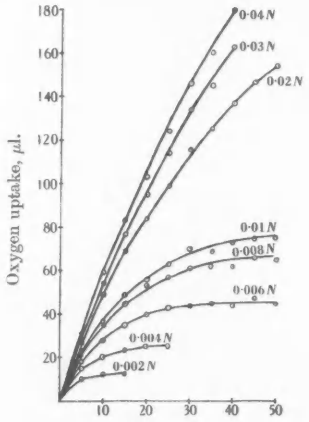


Fig. 6.

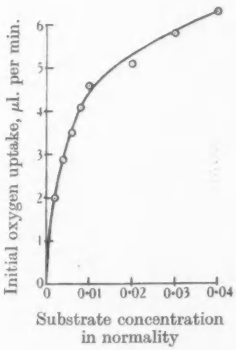


Fig. 7.

Fig. 6. Rate of oxygen uptake by reductone at varying concentrations expressed in normality. Strength of enzyme (undialysed juice), 10 units.

Fig. 7. Initial rate of oxygen uptake by reductone plotted against substrate concentration. Derived from Fig. 6.

As with *d*-gluco-ascorbic acid and reductic acid there was some inhibition as the oxidation of the reductone proceeded (Figs. 1 and 6). The increase in the initial velocity corresponding to the linear portions of the curves (Figs. 2 and 7) with the increase in substrate follows in this graph a form similar to that observed in the *d*-gluco-ascorbic acid and reductic acid graphs. These results which were obtained with undialysed juice were similar to those observed when the dialysed preparation was employed.

In the case of the undialysed juice, when the quantity of enzyme was varied and the concentration of the substrate (0·01 *N*) was kept constant the increase in enzyme at low concentrations brought about a very marked acceleration of the rate of oxidation. As the quantity of enzyme added was increased, however, a stage was reached when a further increase in the enzyme brought about

remarkably little response (Figs. 8 and 9). When dialysed juice was used the rate of O_2 uptake at low enzyme concentrations was greatly reduced (Fig. 10). The curve obtained by plotting initial velocities of O_2 uptake against enzyme

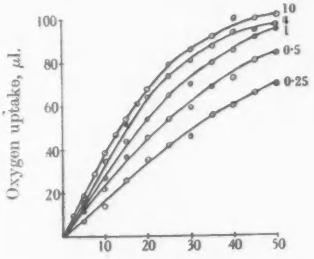


Fig. 8.

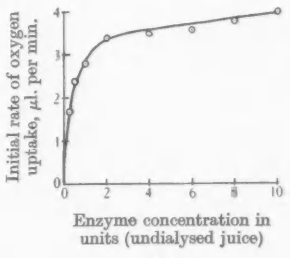


Fig. 9.

Fig. 8. Rate of oxygen uptake by reductone in the presence of varying amounts of *undialysed* cucumber juice. Enzyme concentrations expressed in units. Substrate concentration = 0·01 *N*.

Fig. 9. Initial rate of oxygen uptake by reductone plotted against enzyme concentration. Derived from Fig. 8 and containing some points corresponding to curves which could not conveniently be included in Fig. 8.

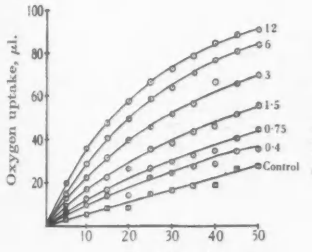


Fig. 10.

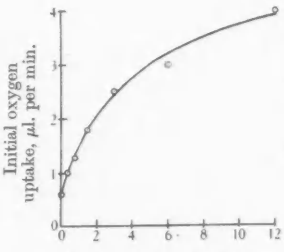


Fig. 11.

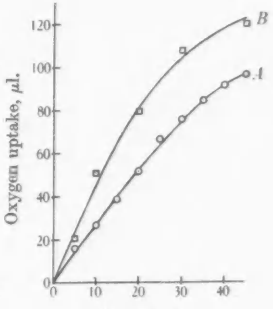


Fig. 12.

Fig. 10. Rate of oxygen uptake by reductone in the presence of varying amounts of *dialysed* cucumber juice. Enzyme concentrations expressed in units. Substrate concentration, 0·01 *N*.

Fig. 11. Initial rate of oxygen uptake by reductone plotted against enzyme concentration. Derived from Fig. 10.

Fig. 12. Comparison of observed oxygen uptake by reductone in the presence of undialysed cucumber juice with that calculated from the disappearance of the substrate. ⊙—⊙ (*A*) observed values. ⊡—⊡ (*B*) calculated values.

concentration (Fig. 11), however, shows that this relationship is again not linear. This behaviour of reductone therefore stands out in striking contrast to that of the other compounds (Figs. 3 and 4).

From Fig. 12 it is seen that the disappearance of the substrate as determined by the method described above was greater than would have been expected

from the amount of O_2 absorbed. The difference is significant since it was found to be reproducible. In this experiment undialysed juice was used but similar results were obtained also with dialysed juice.

Dihydroxymaleic acid as substrate

A commercial sample of dihydroxymaleic acid was purified by recrystallization at 60° from butyl alcohol. The scintillating white crystalline product became powdery on drying *in vacuo* over conc. H_2SO_4. As dihydroxymaleic acid is not very soluble in water it was found convenient to employ its Na salt which was prepared by treating the acid in ethyl alcohol with 2 mol. of sodium ethoxide. The precipitated salt was washed with ethyl alcohol and dried.

The oxidation of dihydroxymaleic acid is characterized by the formation of CO_2, and in order to obviate interference with the measurements of the O_2 uptake the former was absorbed by placing 0·3 ml. of 20 % KOH solution and a roll of filter paper in the central cup of each manometer flask.

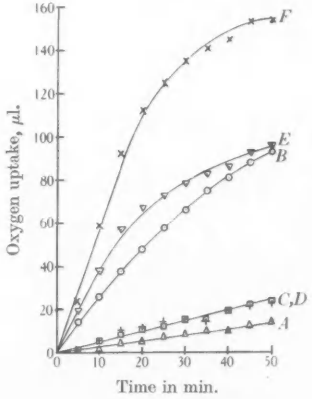


Fig. 13.

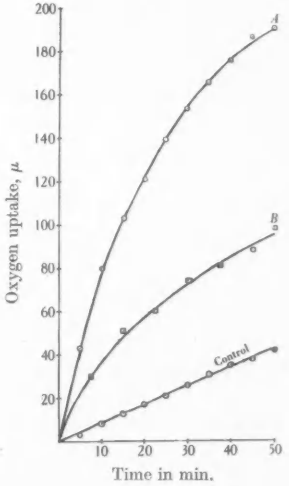


Fig. 14.

Fig. 13. Oxidation of 0·01 *N* dihydroxymaleic acid solutions. △—△ (*A*) with no addition (control). ⊙—⊙ (*B*) in the presence of 1 ml. undialysed cucumber juice. ⊡—⊡ (*C*) in the presence of 1 ml. dialysed cucumber juice. +—+ (*D*) in the presence of 1 ml. dialysed cucumber juice containing 10 mg. *l*-ascorbic acid per 100 ml. ▽—▽ (*E*) in the presence of 1 ml. dialysed cucumber juice and *M*/100 phenol. ×—× (*F*) in the presence of 1 ml. dialysed cucumber juice and *M*/100 catechol.

Fig. 14. Comparison of observed oxygen uptake by dihydroxymaleic acid in the presence of undialysed cucumber juice with that calculated from the disappearance of substrate. ⊙—⊙ (*A*) observed values. ⊡—⊡ (*B*) calculated values.

The action of cucumber juice upon dihydroxymaleic acid solutions differed from that on the preceding substrates in being greatly influenced by dialysis. Whilst the untreated juice caused a considerable O_2 uptake, after dialysis the uptake was hardly more than in the control. Further experiments showed that addition of quantities of ascorbic acid up to 10 mg. per 100 ml. of dialysed juice did not restore its catalytic activity towards dihydroxymaleic acid. On the

other hand, when phenol or catechol was added to the dialysed preparation, so that the final mixtures in the flasks were $M/100$ in respect to these compounds, considerable activity was imparted (Fig. 13).

It is of interest to note that there was a great discrepancy between the volume of O_2 calculated from the disappearance of the substrate in the presence of undialysed juice and the volume of O_2 actually taken up (Fig. 14). This fact, taken in conjunction with the observation made by us that CO_2 was evolved in the process, suggests that the mechanism involved in this oxidation was undoubtedly different from that associated with the enzymic oxidation of the other compounds.

Sulphydryl compounds as substrates

A crystalline sample of glutathione prepared by Pirie's method [1930; 1932] was employed and a commercial sample of cysteine hydrochloride was found to yield satisfactory results. Preliminary experiments have shown that at *p*H 6·0 oxidation was hardly, if at all, accelerated by the presence of the juice at the concentrations used (10 units of enzyme and 0·01 *N* substrate). Further trials have, however, shown that at *p*H 7·4 the oxidation was markedly catalysed and consequently the experiments were carried out at this *p*H. As no quantitative

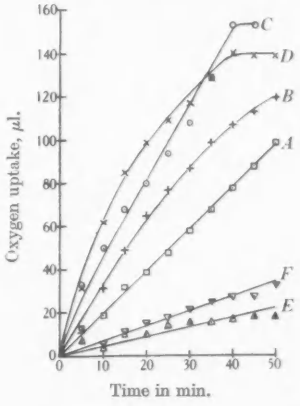


Fig. 15. Oxidation of 0·01 *N* solutions of glutathione and cysteine.

(*a*) In the presence of dialysed cucumber juice — ⊡—⊡ (*A*) GSH. +—+ (*B*) cysteine.

(*b*) In the presence of dialysed cucumber juice with the addition of 10 mg. ascorbic acid per 100 ml. — ⊙—⊙ (*C*) GSH. ×—× (*D*) cysteine.

(*c*) In the absence of enzyme (controls) — △—△ (*E*) GSH ▽—▽ (*F*) cysteine.

assessment of glutathione can be obtained by titration with indophenol, determinations of the substrate concentrations were carried out iodimetrically. The various concentrations of cysteine hydrochloride were made up on the assumption that this compound was pure.

Owing to the fact that undialysed cucumber juice usually contains traces of dehydroascorbic acid, the possibility could not be excluded that the oxidation of the sulphydryl compounds did not take place directly by the enzyme, but was due to the action of dehydroascorbic acid [cf. Hopkins & Morgan, 1936].

Additional experiments were therefore performed with dialysed juice. It will be seen from Fig. 15 that although the addition of 10 mg. of ascorbic acid to 100 ml. of the dialysed juice approximately doubled its oxidizing activity, both sulphydryl compounds were oxidized even in the absence of ascorbic acid at *p*H 7·4; the dialysed juice contained less than 0·1 mg. of dehydroascorbic acid per 100 ml.

A point of interest emerged from these experiments, namely that when different samples of dialysed juice were employed the relative rates of oxidation of the sulphydryl compounds and of *l*-ascorbic acid varied (Figs. 16 and 17).

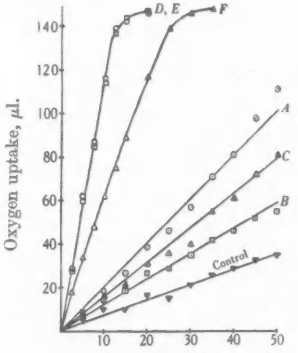


Fig. 16.

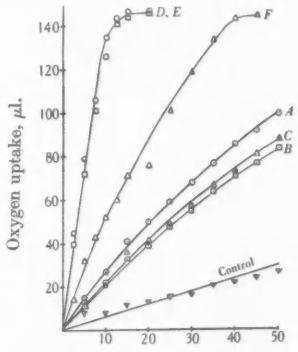


Fig. 17.

Fig. 16. Oxidation of 0·01 *N* solutions of glutathione and *l*-ascorbic acid in the presence of equal amounts of different samples of dialysed cucumber juice.

	GSH	*l*-Ascorbic acid
Sample I	⊙—⊙ (*A*)	○—○ (*D*)
„ II	⊡—⊡ (*B*)	□—□ (*E*)
„ III	△—△ (*C*)	△—△ (*F*)

Fig. 17. Oxidation of 0·01 *N* solutions of cysteine and *l*-ascorbic acid in the presence of equal amounts of different samples of dialysed cucumber juice.

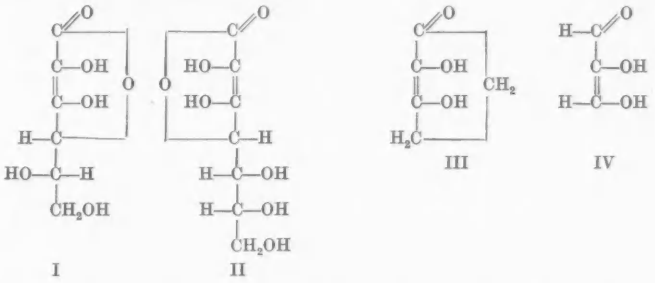
	Cysteine	*l*-Ascorbic acid
Sample I	⊙—⊙ (*A*)	○—○ (*D*)
„ II	⊡—⊡ (*B*)	□—□ (*E*)
„ III	△—△ (*C*)	△—△ (*F*)

This suggests that in all probability different enzymes in the juice were involved in the oxidation of glutathione and cysteine on the one hand, and of *l*-ascorbic acid on the other.

Discussion

The information obtained in this investigation suggests that the action of the ascorbic acid oxidase is not confined to *l*-ascorbic acid (I) and *d*-gluco-ascorbic acid (II), cyclic dienols with an oxygen bridge. It was shown that the oxidations of a cyclic dienol without an oxygen bridge, reductic acid (III) and an acyclic dienol, reductone (IV), both of which were capable of reducing indophenol, were catalysed by the enzyme. In most of the compounds the rate of oxygen absorption showed an inhibition as the oxidation proceeded. The oxidation of *l*-ascorbic acid and, as previously found [Johnson & Zilva, 1937, 2],

that of its analogues with the oxygen ring to the right of the carbon chain, were, however, directly proportional to the time.



I II III IV

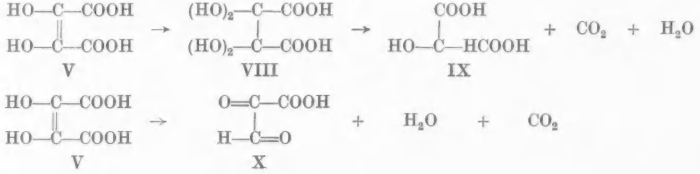
Amongst all the other substrates used in this investigation reductone stood out in its behaviour towards increasing enzyme concentrations in so far that beyond a certain stage the addition of more enzyme brought about very little response in the O_2 uptake (Figs. 9 and 11). The effect was specially noticeable when undialysed juice which showed greater activity towards reductone was employed. A possible explanation of this phenomenon may lie in the fact that reductone can exist in at least two tautomeric forms IV and VI. The high reducing properties of the compound in solution are presumably due to IV which would form the effective substrate and the concentration of which would therefore be controlled by the velocity of the tautomeric conversion. At high enzyme concentrations this may become the limiting factor in the rate of oxidation. The behaviour of reductone towards ascorbic acid oxidase is similar to that of fructose fermented by increasing quantities of yeast as observed by Hopkins & Roberts [1935, 1, 2]. In this case the formation of the fermentable component in the fructose solution could actually be observed by the rate of mutarotation.

In the compounds containing the 5-membered ring the O_2 uptake was consistent with the values calculated from the disappearance of the substrate on the assumption that two atoms of hydrogen were eliminated from each mol. In the case of reductone, however, the O_2 uptake was less than that calculated. This suggests that some of the substrate was utilized in a side reaction. By analogy with the easy conversion of methylglyoxal into lactic acid, the transformation of VI into glyceric acid (VII) provides a mechanism which would be consistent with such an assumption.

VI VII

The oxidation of dihydroxymaleic acid by the cucumber juice, unlike those of reductone and reductic acid, is not due to the presence of ascorbic acid oxidase but to another enzyme system, possibly a phenolase. The high O_2 uptake and the production of CO_2 in this case suggest a breakdown either of the acid or of a primary oxidation product, with the formation of a substance capable of absorbing O_2. It is well known that the products of oxidation of dihydroxymaleic acid depend on the nature of the oxidizing agent. Thus the halogens

oxidize it (V) to dihydroxytartaric acid (VIII) which easily decomposes into hydroxymalonic acid (IX) and CO_2, whilst ferric salts oxidize it to glyoxal-carboxylic acid (X), CO_2 and water. This behaviour of dihydroxymaleic acid towards oxidizing agents provides some suggestion for its more complete degradation in the presence of the juice.



It is of interest to note that the enzymic oxidation of *l*-ascorbic acid *in vivo* has so far been indicated to be due only to the indirect action of a phenolase [Zilva *et al.* 1938] and not to the direct oxidation by the ascorbic acid oxidase. This does not, however, exclude the possibility that the oxidase functions also *in vivo*. The marked capacity of the enzyme to act in dilute solutions of *l*-ascorbic acid as compared with other substrates in fact strongly favours this view, since the vitamin is present in plants in very low concentrations. Even in some of the exceptionally rich sources such as the juice of the mango (Alphonso variety) it is present only to the extent of 0·01 *N*. It would indeed be surprising if the ascorbic acid oxidase were not involved in any of the metabolic functions of the plant.

Summary

Dialysed cucumber juice, apart from its ability to oxidise the ascorbic acid analogues directly, also oxidizes reductone and reductic acid.

Whilst in the compounds containing the five-membered ring the O_2 uptake agrees with the values calculated from the disappearance of the substrate, in the case of reductone the O_2 uptake is less than that calculated. It is suggested that some of the substrate is utilized in a side reaction.

Low concentrations of *l*-ascorbic acid are much more readily oxidized by dialysed juice than *d*-gluco-ascorbic acid, reductic acid and reductone, indicating a greater affinity of the enzyme for the naturally occurring vitamin.

Undialysed, but not dialysed, juice oxidizes dihydroxymaleic acid. Dialysed juice, however, regains its oxidizing activity towards this substrate on addition of catechol or phenol.

In the oxidation of dihydroxymaleic acid there is a formation of CO_2 and a higher O_2 uptake than that calculated from the disappearance of the substrate. The possible mechanism involved in this more complete degradation of the substrate is discussed.

Dialysed cucumber juice is capable of oxidizing glutathione and cysteine. The enzyme responsible for this oxidation appears not to be identical with the ascorbic acid oxidase present in the juice, since the relative rates of oxidation of the sulphydryl compounds and of ascorbic acid vary with different samples of dialysed juice.

We desire to express our gratitude to Prof. E. L. Hirst and to Prof. T. Reichstein for valuable gifts of *d*-gluco-ascorbic acid and of reductic acid. *l*-Ascorbic acid was kindly supplied by Messrs Roche Products, Ltd. One of us (G. A. S.) is indebted to the Medical Research Council for a whole time grant.

REFERENCES

Euler & Klussmann (1933). *Ark. Kemi Min. Geol.* **11** B, no. 8.
—— & Martius (1933, 1). *Ark. Kemi Min. Geol.* **11** B, no. 12
—— —— (1933, 2). *Ark. Kemi Min. Geol.* **11** B, no. 14.
—— —— (1933, 3). *Svensk kem. Tidskr.* **45**, 73.
—— —— (1933, 4). *Liebigs Ann.* **505**, 73.
Hopkins, F. G. & Morgan (1936). *Biochem. J.* **30**, 1446.
Hopkins, R. H. & Roberts (1935, 1). *Biochem. J.* **29**, 931.
—— —— (1935, 2). *Biochem. J.* **29**, 2486.
Johnson & Zilva (1937, 1). *Biochem. J.* **31**, 438.
—— —— (1937, 2). *Biochem. J.* **31**, 1366.
Keilin & Mann (1938). *Proc. roy. Soc.* B, **125**, 187.
Pirie (1930). *Biochem. J.* **24**, 51.
—— (1932). *Biochem. J.* **26**, 75.
Reichstein & Oppenauer (1933). *Helv. Chim. Acta*, **16**, 988.
—— —— (1934). *Helv. Chim. Acta*, **17**, 390.
Szent-Györgyi (1930). *Science*, **72**, 125.
—— (1931). *J. biol. Chem.* **90**, 385.
Zilva (1934). *Biochem. J.* **28**, 633.
—— (1935, 1). *Biochem. J.* **29**, 1612.
—— (1935, 2). *Biochem. J.* **29**, 2366.
—— Kidd & West (1935). *Report of Food Investigation Board*, p. 110.
—— —— —— (1938). *New Phytol.* **37**, 345.

CCXLIX. UTILIZATION OF POLY-GLYCEROL ESTERS

By M. BODANSKY, CORNELIUS L. HERRMANN and KATHERINE CAMPBELL

From the John Sealy Memorial Research Laboratory and the Department of Pathological Chemistry, University of Texas School of Medicine, Glaveston, Texas

(*Received 30 August 1938*)

The polymerization of glycerol to di- and tri-glycerol and to more highly polymerized glycerols has been described by a number of investigators, including Harris [1935], who has also prepared mixed esters of these polyglycerols with fatty acids. The large variety of esters which may be thus synthesized, though in many respects resembling fats, are distinguished by their wetting, emulsifying and other capillary-active properties, these being apparently related to the structure of the molecule in that it has a lipophilic group, in the form of a long-chain fatty acid residue, and hydrophilic groups in the form of free hydroxyl at or near an end of the molecule. These surface-active functions of the polyglyceride esters are being utilized in various industries, and Harris [1933] describes their mechanism and suggests the use of these esters as emulsifying agents in edible emulsions. He also suggests their use in small percentages in margarine with the object of reducing the spattering of the margarine during frying. Because of the uniqueness of this group of compounds and their utility, it seemed of interest to investigate their utilization in the animal organism, the present report being principally concerned with growth and faecal excretion of fat.

Experimental

Albino rats were used, the stock in this laboratory being a pure inbred Wistar strain, reared under favourable conditions. The basic diet and the one fed to the controls consisted of ground Purina chow, to which was added a dried milk preparation (5%), yeast (1%) and water to proper consistency. For the experimental animals the basic ration was supplemented either with lard (5 or 10%), or polyglycerol ester (5 or 10%). In one large series of experiments the diet was begun soon after weaning (25–30 days old) and continued for as long as 14 months. Observations on over 100 rats revealed that both in the lard and polyglycerol ester-fed rats the rate of growth was normal or better than normal, the criterion for normal growth being that of the controls, which in our experience exceeded somewhat the standards obtained for the Wistar colony, as recorded by Greenman & Duhring [1931].

Generally the lard-fed rats attained somewhat greater weights than the rats in the polyglycerol ester group, the difference being apparently related to the much greater accumulation of fat in the depots (perirenal, mesenteric, omental, genital etc.) in the former. Histological examination of the tissues disclosed no abnormalities in either of the experimental groups. Rats were maintained successfully on the polyglycerol ester and lard diets through three generations,

the diets being continued without interruption throughout, including the periods of gestation and lactation. A number of typical growth curves of first generation rats fed with polyglycerol ester and lard are given in the accompanying charts.

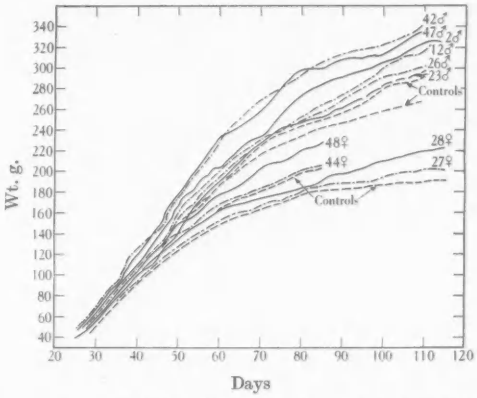


Fig. 1. Growth curves of rats: solid lines, 10% lard; dots and dashes, 10% polyglycerol ester; broken lines, controls.

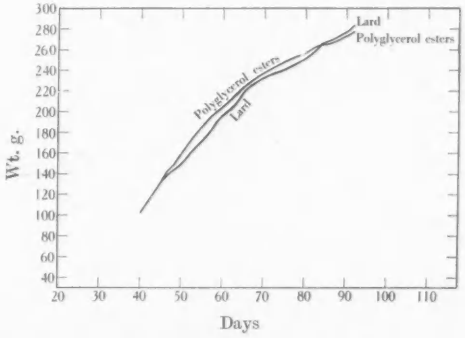


Fig. 2. Composite growth curves of three rats fed polyglycerol ester and three which received a comparable amount of lard (10% of diet). The six rats were male litter-mates and the offspring of parents reared on a polyglycerol ester-containing diet.

The fatty acids of the polyglycerol preparation used in these studies were chiefly stearic and oleic. The compound had I.V. 31·6 and sap. equiv. 143. Its utilization was determined in a series of mature male and female rats as indicated in Table I. During the experimental period of 24 days a record was kept of the daily food consumption. The faeces for each 3-day interval were collected separately and weighed, the excreta for eight such periods being combined and sampled for analysis. In the control group the data are based on 21 days of observation, as one 3-day specimen was discarded owing to incomplete collection. There were no striking differences in the water contents of the faeces of the various groups. It is to be noted further that the ratio of food consumed to the weight of faeces varied within narrow limits and was not significantly

Table I. *Utilization of fat in rats receiving supplements of lard or polyglycerol ester*

Exp.	Duration days	Rats no. and sex	Initial and final wt. g.	Food consumed g.	Faeces g.	Faeces % of food consumed	Faecal fat %	Faecal fat I.V.	Depot fat I.V.
Lard, 5%	24	45 ♂	369–390	1398	224	16·0	3·96	76·4	76·6
		46 ♂	410–448						75·5
		64 ♀	215–240	961	172	17·9	2·29	89·5	77·3
		65 ♀	209–232						
Lard, 10%	24	25 ♂	333–340	1018	178	17·5	3·82	78·7	—
		26 ♂	330–338						
		3 ♀	225–245	812	127	15·6	2·30	87·2	78
		28 ♀	227–208						
Polyglycerol ester, 5%	24	62 ♂	273–322	1164	194	16·6	4·40	66·6	—
		63 ♂	290–339						
		35 ♀	222–225	857	157	18·3	2·63	81·2	78·1
		5 ♀	253–256						
Polyglycerol ester, 10%	24	21 ♂	356–360	1047	166	15·8	6·26	58·3	68·8
		23 ♂	309–348						
		13 ♀	193–210	704	111	15·8	3·41	63·8	76·6
		14 ♀	172–200						78·4
Control	21	51 ♂	332–358	925	126	13·5	1·66	77·3	—
		52 ♂	322–332						
		39 ♀	216–227	865	145	16·8	2·24	91·2	67·3
		32 ♀	213–225						

different in the lard and polyglycerol ester groups. In the rats receiving polyglycerol ester, the amounts of faecal lipins were somewhat greater than those in the lard group, which in turn exceeded the values found in the controls. However, even if all the ether-soluble material of the faeces in rats 21, 23, 13 and 14 represented unabsorbed polyglycerol compound, the results would nevertheless indicate 90% utilization in the males and 94·62% in the females. But judging from the faecal fat in the controls as well as the I.V., it is conservative to estimate that at least 95–98% of the polyglycerol esters of the rations were digested and absorbed even when they constituted 10% of the food mixture.

Polyglycerol esters of oleic and linoleic acids. The observation by Hansen [1933] that in infantile eczema the serum fatty acids have an abnormally low I.V. and his subsequent statement [1933–4] concerning the therapeutic action of unsaturated oils, such as linseed oil, stimulated in us further interest in the problem. It seemed that if unsaturated fatty acids actually exercised a beneficial effect their administration in polyglycerol combination would be of advantage, if for no other reason than the ease of preparing suitable emulsions. Accordingly, polyglycerol was esterified with commercial oleic acid and with a good grade of linseed oil. The constants for oleic acid ester were: I.V. 69·4, sap. equiv. 151. Those for the linseed oil fatty acid ester mixture were 116·7 and 137·2, respectively. The esters were fed at levels of 5% as indicated in Table II. The controls were maintained on the basal ration, while for further comparison other groups of rats received butter, 10%; oleomargarine, 10%; linseed oil, 5%; cocoa butter, 5%. As we were especially interested in the utilization of linseed oil and the polyglycerol esters of the unsaturated fatty acids in the young, the rats selected for this work were not more than 40 days old (oleic and linoleic ester groups and the controls, Table II, were 30 days old at the beginning of the experiment). It will be noted that the rate of growth was normal, or somewhat

Table II. *Utilization of various fats, including polyglycerol esters of oleic and linoleic acids*

Exp.	Duration days	Rats no. and sex	Initial and final wt. g.	Food consumed g.	Faeces g.	Faeces % of food consumed	Faecal fat %	Faecal fat I.V.	Depot fat I.V.	Depot fat Sap. equiv.
Oleic ester, 5%	24	68 ♂	56–160	971	157	16·2	4·45	86·5	73·5	216·5
		69 ♂	54–158						80·5	205·5
		70 ♀	53–142	783	116	14·8	4·9	92·3	76·9	—
		71 ♀	50–144							
Linoleic ester, 5%	24	72 ♂	58–162	932	124	13·3	3·79	77·6	81·2	198·5
		73 ♂	56–152							197·5
		74 ♀	45–128	737	132	17·9	4·34	87·2	91·5	—
		75 ♀	50–126						81·1	
Control	24	76 ♂	52–159	945	160	17·05	4·14	80·5	73·2	199·5
		77 ♂	55–160							
		78 ♀	56–137	927	191	20·6	2·83	89·7	81·2	202
		79 ♀	58–148							
Butter, 10%	21	80 ♂	67–151	878	125	14·3	4·16	57·0	76·5	201
		81 ♂	75–165							
		82 ♀	64–140	827	138	16·7	3·58	72·5	65·1	199
		83 ♀	63–124							
Oleomargarine 10%	21	86 ♀	53–128	710	127	17·6	2·94	73·0	79·6	198·5
		87 ♀	60–143							
		89 ♂	76–163	874	137	15·7	3·59	72·1	94·0	192·5
		90 ♂	78–174							
Linseed oil, 5%	24	91 ♀	73–132	545	107	19·6	4·18	78·2	81·5	182·5
		92 ♀	75–140							
		93 ♂	70–150	944	152	16·1	5·11	65·1	82·8	—
		94 ♂	78–180							
Cocoa butter, 5%	24	95 ♀	64–118	748	117	15·7	4·54	67·0	79·9	203·5
		96 ♀	74–149							

better than normal in all groups (note especially the excellent growth of the polyglycerol ester groups). As shown by the data for faecal lipins, there was almost complete utilization of the polyglycerol esters of oleic and linoleic acids, as well as of the linseed oil and other fats used. Under the conditions of the experiments, the composition of the depot fat was only slightly influenced by the kind of fat incorporated in the diet.

The faeces were analysed essentially according to the method of Sperry & Bloor [1924], while for the analysis of the tissues the methods of Reed and associates [1930; 1932] were used.

Comment

The composition of the "fat" of the faeces, while not altogether unrelated to that of the fat consumed, is remarkably constant and independent of the diet, as has been shown by a number of investigators [Hill & Bloor, 1922; Holmes & Kerr, 1923–4; Sperry & Bloor, 1924]. Only under conditions in which digestion and absorption of fat are diminished, whether through disease or otherwise, does the composition of the faecal fat reflect that of the diet. Accordingly, analysis of the faeces may afford an accurate measure of the utilization of a fat in the alimentary tract. In the light of existing knowledge the results of the present study demonstrate that the coefficients of digestibility (including absorption) of the polyglycerol esters of stearic, oleic and linoleic

acids are essentially the same as those of naturally occurring fats (compare with data of Holmes & Kerr [1923–4]. *In vitro* experiments have confirmed the digestibility of these esters by lipase.

SUMMARY

Compounds produced by partial esterification of the polymerides of glycerol (diglycerol, triglycerol, tetraglycerol etc.) with fat-forming fatty acids, when fed at levels of 5 and 10% of the diet were found to be as well utilized in the alimentary tract of the albino rat as were naturally occurring fats. No untoward effects, as regards growth, reproduction, lactation and the microscopic appearance of the tissues, were observed as the result of long-continued feeding of these compounds.

Acknowledgement is due to Messrs Harris and Epstein for suggesting the problem and to the research staff of The Emulsol Corporation of Chicago for the synthesis of the compounds used in this work.

REFERENCES

Greenman & Duhring (1931). Breeding and Care of the Albino Rat for Research Purposes. Publication of the Wistar Institute of Anatomy and Biology, Philadelphia.
Hansen (1933). *Proc. Soc. exp. Biol., N.Y.*, **30**, 1198.
—— (1933–4). *Proc. Soc. exp. Biol., N.Y.*, **31**, 160.
Harris (1933). U.S. Patent No. 1,917,257.
—— (1935). U.S. Patents No. 2,022,766 and No. 2,023,388.
Hill & Bloor (1922). *J. biol. Chem.* **53**, 171.
Holmes & Kerr (1923–4). *J. biol. Chem.* **58**, 377.
Reed, Yamaguchi, Anderson & Mendel (1930). *J. biol. Chem.* **87**, 147.
—— —— —— —— (1932). *J. biol. Chem.* **96**, 313.
Sperry & Bloor (1924). *J. biol. Chem.* **60**, 261.

CCL. A NOTE ON THE DEXTRAN PRODUCED FROM SUCROSE BY *BETACOCCUS ARABINOSACEOUS HAEMOLYTICUS*

By MAURICE STACEY and FREDERICK ROBERT YOUD

From the A. E. Hills Laboratories, University of Birmingham

(*Received 14 September 1938*)

During the large scale production of the dextran from sucrose by *Betacoccus arabinosaceous haemolyticus* (*Leuconostoc dextranicum*) using the method described previously by Carruthers & Cooper [1936], unforeseen and inexplicable irregularities of growth occurred, so that a further investigation of the metabolic requirements of the organism was deemed necessary. It has been possible to develop a more satisfactory medium for the dextran production. In this medium use is made of commercial maple syrup to provide a reliable supply of the "accessory growth substance" (which may be simply a source of available nitrogen) together with an increase to 20% in the concentration of sucrose.

Sufficiently large quantities of the dextran to facilitate its chemical examination were isolated and it was purified without the use of heat, acid or alkali. Previous difficulties in removing protein impurities were minimized by a preliminary filtration of the dextran solution through a kieselguhr pad. In purified samples, despite the fact that the usual protein tests were negative, a small fragment of nitrogenous material (N, 0·5% approx.) persisted. The dextran could be obtained N-free only after mild acid or alkaline treatment. Complete elimination of the nitrogenous constituent was accompanied by a decrease in viscosity of the dextran. This phenomenon was especially marked when a solution was treated for a short period at 100° with weak acid. In such a case the nitrogenous constituent separated from the clear solution in the form of a flocculent precipitate—a phenomenon which we have frequently observed with other polysaccharides of microbiological origin, notably with Types I and II pneumococcus specific polysaccharides.

In the case of the dextran produced by *Leuconostoc mesenteroides*, Fitzgerald [1933] found that its immunizing properties, originally investigated by Zozaya [1932, 1], were due entirely to a nitrogenous constituent since all antigenic activity disappeared when the N content of the dextran fell below 0·2%. In a similar manner certain antigenic properties ascribed to gum arabic have also been shown to be connected with small amounts of a nitrogenous constituent [Uhlenhuth & Remy, 1935]. The significant decrease in viscosity of the dextran after removal of its nitrogenous fraction may indeed indicate that the latter, although comparatively small, is an actual constituent of the dextran macromolecule. The possible significance of such a compound acting as a stabilizer of the macromolecular state of the polysaccharide, and thereby conserving certain of its antigenic properties, may be worthy of investigation. In this connexion it is of interest to recall the experiments of Zozaya [1932, 2] who claimed to have endowed otherwise inactive polysaccharides with antigenic activity by injecting them as a suspension in collodion.

The relationship between the acetyl content of dextrans and their antigenic properties is under investigation in another Laboratory [Fowler *et al.* 1937],

so that it is of interest to report that the dextran described in this paper and isolated under the least drastic conditions contained no acetyl residues.

A preliminary account of the structure of the dextran has recently been published from these laboratories [Peat *et al.* 1938]. It has been established that this polysaccharide is constituted of a linear chain of several hundred glucose units mutually linked through the 1:6-positions. This is the gentiobiose linkage Further details of the structural investigations will be published later.

Experimental

Following the method of Carruthers & Cooper [1936] for dextran production it was observed that in some flasks growth and viscosity were readily produced whilst in others, prepared in an apparently identical manner, little or no growth took place. The irregularity became particularly marked when the volume of metabolism solution was increased beyond 100 ml. and also after repeated subculturing of the organism. In an investigation into the cause of this phenomenon the following points emerged.

(1) The *p*H values of the medium were identical in both viscous and weak cultures during and after growth, indicating that inhibition of dextran formation was not due to acid production.

(2) Sterilization of sucrose and peptone solutions separately, followed by aseptic mixing before inoculation, gave increased yields of dextran but growth was still irregular.

(3) Malt extract, lucerne root extract and yeast-water failed to provide a growth stimulant.

(4) Molasses extract and Demarara sugar gave inconsistent results and, moreover, when the dextran was formed it was heavily pigmented and difficult to purify.

(5) A medium containing 20% sucrose and 5% commercial maple syrup gave satisfactory results. The dextran formed in this case was free from pigmented material and was readily purified.

Large-scale dextran production

Peptone solution (double strength) and sucrose solution (40 %) containing maple syrup (5 %), were steamed separately on two successive days and then mixed aseptically in amounts of 100 ml. of each in 500 ml. conical flasks. After a third steaming the flasks were cooled and inoculated heavily, using sterile pipettes, with a viscous 48-hr.-old culture of *L. dextranicum*. After incubation at 30° for 10 days the viscous opalescent solution was filtered through cotton wool. The dextran was precipitated in the form of a white powder by the addition of 2 vol. of ethyl alcohol. It was filtered, washed with alcohol and dried *in vacuo*.

The crude material thus isolated amounted to 25% of the sucrose employed. It contained approx. 10% of bacterial protein the greater part of which was removed by repeated filtration of a dilute aqueous solution through kieselguhr. Final traces of protein material were removed by alcoholic fractionation, which, moreover, revealed that the dextran was essentially homogeneous. The purified dextran had the following properties: $[\alpha]_D^{20°}$ +180° in water (*c*, 1·0); ash, 0·4 %; N, 0·25 %; moisture, 8 %. The small amount of nitrogeneous constituent could not be removed by fractionation and its presence was characterized by a faint opalescence of an aqueous solution of the dextran. On heating such a solution with *N*/10 acetic acid or *N*/10 HCl a small amount of flocculent precipitate

separated and the opalescence disappeared. This treatment, as shown below, caused a considerable decrease in viscosity of the dextran:

	$[\alpha]_D$	Rel. vis. in water (1% sol.)	Rel. vis. in saline (1% sol.)	N %	Reduction to Fehling's solution
Normal dextran	+180°	3·20	4·40	0·25	None
Dextran heated with $N/10$ HCl at 100° for 15 min.	+180°	1·01	1·15	0·0	None

Hydrolysis of the dextran was carried out using N H_2SO_4 at 100° for 18 hr., 5·0 g. yielding 5·6 g. of crystalline *d*-glucose. No other sugar was isolated.

Summary

A reliable method is described for the large-scale production of dextran by *Leuconostoc dextranicum* and the purification and properties of the dextran are recorded.

The authors are indebted to Prof. W. N. Haworth and to Dr Stanley Peat for their interest in this work.

References

Carruthers & Cooper (1936). *Biochem. J.* **30**, 1001.
Fitzgerald (1933). *Trans. Roy. Soc. Can.* **27**, 1.
Fowler, Buckland, Brauns & Hibbert (1937). *Canad. J. Res.* B, **15**, 486.
Peat, Stacey & Schlüchterer (1938). *Nature, Lond.*, **141**, 876.
Uhlenhuth & Remy (1935). *Z. Immum. Forsch.* **85**, 3283.
Zozaya (1932, 1). *J. exp. Med.* **55**, 353.
—— (1932, 2). *J. exp. Med.* **55**, 351.

CCLI. INVESTIGATION OF A POLYSACCHARIDE PRODUCED FROM SUCROSE BY *BETA-BACTERIUM VERMIFORMÉ* (WARD-MEYER)

By WALTER DONALD DAKER and MAURICE STACEY

From the A. E. Hills Laboratories, University of Birmingham

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We have been able to examine a polysaccharide formed from sucrose by an organism (*Betabacterium vermiformé*) recently studied by Prof. A. J. Kluyver, to whom we are greatly indebted for a culture. The organism was isolated by Mr H. D. Meyer (private communication) in the course of an extensive and careful bacteriological examination of "Tibi-complex". The latter originates in Mexico where it occurs in the form of hard granules on the disk-shaped leaves of the *Opundia* plant. It has long been used by the natives for the preparation of a beverage by fermentation and is analogous to the classic example, the Kephir yeast of the Arabs. As in the case of Kephir, Tibi fermentation is due to the symbiotic action of a yeast and a bacterium. The bacterium isolated by Meyer appeared to be identical with that isolated by Ward [1892] from the Ginger-beer plant.

On a sucrose-gelatin medium, the bacterium readily formed colonies surrounded by a highly viscous secretion and its growth on gelatin slopes closely resembled that of *Betacoccus arabinosaceous anhaemolyticus* (*Leuconostoc mesenterioides*). Meyer was able to show that the viscous secretion consisted mainly of a polyglucose with properties strikingly similar to those of the dextran synthesized by *B. arab. anhaemolyticus* [Tarr & Hibbert, 1931]. We have been able to confirm the finding that it is indeed a dextran, resembling closely in physical properties the dextrans from both *B. arab. haemolyticus* and *B. arab. anhaemolyticus*.

Our preliminary investigations were concerned with production of the polysaccharide in a liquid medium. The medium adopted for preparing the dextran from *B. arab. haemolyticus* [Stacey & Youd, 1938] was unsuitable for the growth of *Betabact. vermiformé* although when a satisfactory substrate for the latter was devised certain similarities in the growth of the two organisms were observed.

Both dextrans could be synthesized from sucrose only; moreover, in each case it was mainly the glucose constituent of the sucrose molecule which appeared to be utilized in the growth. For consistent dextran production both organisms required a source of available nitrogen which was best supplied by maple syrup to *B. arab. haemolyticus* and by yeast extract to *Betabact. vermiformé*. The polysaccharide could conveniently be prepared in yields amounting to 20% of the sucrose employed in a simple yeast extract-sucrose medium. The growth reached a maximum in about 10 days and was quite uniform even in large volumes of liquid—a property which facilitated the large-scale dextran production.

Separation of the polysaccharide was effected by alcoholic precipitation followed by washing and drying by the usual methods. The crude material contained approx. 0·2% N and was insoluble in water. This insolubility differentiates it from the *Leuconostoc* dextrans which are readily soluble in water.

During growth a large proportion of the dextran became insoluble in the metabolism solution and after isolation and drying it could not be dissolved even in boiling water. On dissolving this insoluble material in strong acid or alkali about 5% of bacterial "debris" separated and was removed in a centrifuge. Thereafter the dextran, precipitated by alcohol, was now water-soluble, N-free, showed $[\alpha]_D + 180°$ in water and was indistinguishable in physical properties from the *Leuconostoc* dextrans.

From the products of acid hydrolysis, crystalline glucose (in 93% yield) was the only sugar isolated. Structural investigation has revealed that the main part of the molecule is constituted of α-glucose units mutually linked through the 1:6-positions, i.e. the linkage which obtains in gentiobiose [cf. Peat *et al.* 1938]. These and other investigations will be described later.

Experimental

Stock cultures of *Betabacterium vermiformé* received from Prof. A. J. Kluyver were maintained on gelatin slopes of the following composition: KCl, 0·5%; Na_2HPO_4, 0·2%; sucrose, 10%; gelatin, 15%; yeast-water, 10%.

In a search for optimum conditions for dextran production in liquid media, the following main facts were established.

(1) The organism could synthesize the dextran from sucrose only, the yield increasing to a maximum at a sucrose concentration of 20%. Complete inhibition of growth occurred when the sucrose was replaced by glucose, fructose, maltose, lactose, glycerol, mannitol, sorbitol, inulin or laevan.

(2) No dextran formation took place in a sucrose-peptone medium in the absence of other sources of N the best of which was 10% yeast-water extract. Addition of maple syrup or beet sugar molasses gave unsatisfactory dextran production in the absence of an extra N supply, but in the presence of peptone (0·1%) or asparagine (0·1%) production was good.

(3) Tomato extract and a crude vitamin B concentrate were able also to provide the source of available N.

(4) The yield of dextran from a 10% yeast-water-sucrose medium was slightly increased by the presence of urea (0·1%) or succinamide (0·1%) or creatinine (0·1%). Glycine, *l*-leucine, malonamide and *d*-alanine appeared to act as growth inhibitors.

(5) During growth the *p*H of the metabolism solution was unchanged so that addition of $CaCO_3$ offered no advantage.

(6) No dextran was produced when the organism was grown in the medium which had proved satisfactory for the production of dextran from *Leuconostoc dextranicum*.

(7) The volume of metabolism solution did not materially affect the rate or amount of dextran formed in a suitable medium.

Large-scale production

Satisfactory results were obtained with the following medium: yeast-water, 10%; KCl, 0·5%; Na_2HPO_4, 0·2%; sucrose, 20%. The medium was distributed in 600 ml. quantities in 1-litre conical flasks and steamed on three successive days. The flasks were inoculated, using small sterile pipettes, from a viscous 48 hr.-old culture of *Betabact. vermiformé* growing in test tubes in an identical medium. After incubation at 30° for several days the medium became very viscous and a dense gummy layer was formed at the bottom of each flask. (In preliminary experiments this material was isolated separately by decantation and was shown to consist of crude dextran.) After 10 days' incubation the

contents of the flasks were well shaken and run through cotton wool into 2 vol. of alcohol.

The crude dextran which was thrown down was washed repeatedly with alcohol, ground to a fine powder in a mortar and dried in a vacuum. The average yield amounted to 20% of the sucrose used. A considerable amount of fructose was isolated from the mother liquors [cf. Carruthers & Cooper, 1936] indicating that the dextran was probably synthesized from the glucose moiety of the sucrose molecule.

Purification and properties

The crude dextran (N 0·2%) was completely insoluble in water and did not swell in boiling water. It dissolved readily in 5*N* NaOH and in 10*N* HCl and showed optical properties as follows: $[\alpha]_D^{20°}+177°$ in 10*N* HCl (*c*, 0·68); $[\alpha]_D+179°$ in 5*N* NaOH (*c*, 0·90). It was rendered soluble in water by the following method suggested by Dr F. Smith: dextran (3 g.) was stirred vigorously in cold conc. HCl (20 ml.) and when evenly suspended was centrifuged. A small amount of amorphous material was thrown down and the clear supernatant liquid was poured into excess of ethyl alcohol. The dextran was precipitated in the form of a fine white powder which was washed free from acid by alcohol and ether and dried in a vacuum. Yield, 2·8 g.

The product was now readily soluble in water. It had no reducing action on Fehling's solution, contained no N and its specific rotation was unaltered by the acid treatment. $[\alpha]_D^{20°}+175°$ in 10*N* HCl (*c*, 1·0); $[\alpha]_D^{20°}+177°$ in water (*c*, 1·3); $[\alpha]_D+179°$ in 5*N* NaOH (*c*, 0·95).

The insoluble gummy layer usually found at the bottom of the metabolism solution and mentioned previously, was treated with conc. HCl in the manner described and yielded a product, $[\alpha]_D^{20°}+179°$ in 5*N* NaOH (*c*, 1·3), identical with that in the metabolism solution. An average sample of dextran had the following properties: $[\alpha]_D^{20°}+180°$ (*c*, 1·3); ash, 0·23%; I.V. 4·7. On hydrolysis with *N* H_2SO_4 at 100° the following polarimetric changes were observed: $[\alpha]_D^{20°}+183°$ (0 min.), +148° (45 min.), +142° (80 min.), +132° (115 min.), +117° (190 min.), +98° (310 min.), +85° (370 min.), +79° (430 min.), +66° (490 min.), +54° (925 min., equilibrium value).

From the hydrolysate crystalline *d*-glucose, $[\alpha]_D+52·4°$, was isolated in 93% yield. The chemical investigation of the dextran will be reported at a later date.

SUMMARY

A practicable method is described for the preparation of a new polysaccharide from sucrose by means of an organism *Betabacterium vermiformé* (Ward-Meyer). The polysaccharide shows a close resemblance in properties to the dextrans synthesized by the *Leuconostoc* species.

The authors are indebted to Prof. A. J. Kluyver for suggesting this investigation and for providing cultures, and they also wish to express their thanks to Prof. W. N. Haworth and to Dr Stanley Peat for their interest in the work.

REFERENCES

Carruthers & Cooper (1936). *Biochem. J.* **30**, 1001.
Peat, Schlüchterer & Stacey (1938). *Nature, Lond.*, **141**, 876.
Stacey & Youd (1938). *Biochem. J.* **32**, 1943.
Tarr & Hibbert (1931). *Canad. J. Res.* **5**, 414.
Ward (1892). *Philos. Trans.* **183**, 126.

CCLII. POLYLAEVANS FORMED BY THE CARBOHYDRATE METABOLISM OF CERTAIN BACTERIA

By STIG VEIBEL

From the Chemical Laboratory of the University, Copenhagen

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ØRSKOV [1930; 1931; 1936; 1938; Ørskov & Poulsen, 1931] has stated that when certain Gram-negative organisms found in milk and certain soil bacteria (*Actinomycetes*) are grown on sucrose- or raffinose-containing subtrates, polysaccharides are formed abundantly. The substances formed have, however, not been examined chemically, and their nature as polysaccharides has been deduced from their appearance alone. It was therefore thought to be of interest to examine them more thoroughly, and the investigation here reported shows that the three micro-organisms hitherto studied do in fact form polylaevans and most probably the same laevan in each case.

(1) *Substance from milk bacteria [Ørskov, 1931]*

The micro-organisms were grown on 2% sucrose-agar. After 2 days the bacteria were separated by filtration and the "colonies" of polysaccharide remaining in the somewhat opalescent liquid were precipitated by addition of alcohol and ether. The supernatant liquid was decanted and the precipitate was washed with physiological salt solution, separated by centrifuging and dried to constant weight in a vacuum desiccator over conc. H_2SO_4.

Analysis showed that the substance isolated had the composition of a polysaccharide, contaminated with a small amount of protein possibly originating from residual bacteria or from cellular nuclei in the "colonies" of polysaccharide. (Found: C, 44·32; H, 6·41; N (Kjeldahl), 0·59%. $(C_6H_{10}O_5)_n$ requires C, 44·42; H, 6·23%.) The polysaccharide is practically insoluble in all solvents. It does not reduce Fehling's solution, but after hydrolysis with boiling dilute acid it gives strongly reducing products.

0·54 g. of the polysaccharide was heated for 3 hr. on the steam bath with 40 ml. water and 5 ml. *N* HCl. The solution was left at room temperature overnight; next day it was made up to 50 ml. in a measuring flask, filtered from a trace of flocculent precipitate and its optical rotation determined; $\alpha_D^{20°}$ was $-1·77°$ (c (calc. as monosaccharide) $= 1·2$, $l = 2$), whence $[\alpha]_D^{20°} = -74°$. This is a minimum value and it is some 20% lower than the specific rotation of equilibrium fructose, but as already pointed out the polysaccharide was not N-free; it may therefore be assumed with some probability that the polysaccharide is a (slightly impure) fructose anhydride (laevan).

In order to confirm this supposition, a solution of 1·5 g. polysaccharide was neutralized with NaOH, concentrated in a vacuum at 35–40° to low volume and finally dried in a vacuum desiccator over H_2SO_4. The residue, 1·2 g., was treated with dry acetone and conc. H_2SO_4 as recommended by Ohle & Koller [1924] for the preparation of β-diacetonefructose. 0·9 g. of a compound with M.P. 95–96° and $[\alpha]_D^{20°} = -38·6°$ (alcohol, $c = 1·166$) or $-34·3°$ (water, $c = 1·808$) was obtained. Ohle & Koller [1924] obtained from 10 g. of fructose 6·5 g. of diacetonefructose

with M.P. 97° and $[\alpha]_D^{20°} = -36{\cdot}69°$ (alcohol, $c = 1{\cdot}172$) or $-26{\cdot}17°$ (water, $c = 1{\cdot}856$); they indicate, however, that the specific rotation in aqueous solution depends on the concentration; for $c = 3{\cdot}161$ they found $[\alpha]_D^{20°} = -32{\cdot}9°$.

For comparison we prepared β-diacetonefructose from authentic fructose. From 2·5 g. we obtained 1·4 g. of a preparation with M.P. 96–97°, i.e. nearly the same yield as that quoted by Ohle & Koller. The specific rotation of this preparation in aqueous solution did not depend on the concentration: for $c = 3{\cdot}193$ $[\alpha]_D^{20°}$ was $-34{\cdot}0°$ and for $c = 1{\cdot}811$ $[\alpha]_D^{20°}$ was $-34{\cdot}1°$, in agreement with Ault *et al.* [1935] who found $[\alpha]_D^{21°} = -34°$ (water, $c = 0{\cdot}9$). For an alcoholic solution with $c = 1{\cdot}183$ we found $[\alpha]_D^{20°} = -37{\cdot}97°$ for the product from fructose. The values found by us for authentic diacetonefructose are thus identical with those found for the acetone derivative of the product of hydrolysis of the polysaccharide, which therefore may be assumed to be fructose.

Further confirmation of this assumption has been obtained by the method of Bridel [1930] for detection of glucose or galactose in plant materials, namely, solution of the substance in 70% methyl alcohol and examination of the influence of addition of emulsin (β-glucosidase). If a sugar, which is able to form a methyl glycoside under the influence of emulsin is present, an alteration of the optical rotation of the solution will take place.

The monosaccharide obtained from 4·65 g. of the polysaccharide was dissolved in 100 ml. 70% methyl alcohol and 0·2 g. of an emulsin preparation with sal. f. 0·091 was added. The solution was rotated mechanically for 14 days at 30°. Before addition of emulsin the solution had $\alpha_D - 5{\cdot}17°$ and the rotation remained unaltered during the experiment. From this it may be concluded at least that glucose and galactose are absent from the solution, and the assumption that fructose is the sole product of hydrolysis gains in probability.

(2) *Substance from soil* Actinomycete, *strain 38*. [*Ørskov, 1938*]

The bacteria were grown as above. The polysaccharide formed in this case, although corpuscular, did not form "colonies"; it was isolated in the same manner as previously. 2 g. were hydrolysed by heating on the steam bath for 3 hr. with 150 ml. water and 5 ml. *N*/10 HCl. The solution was left overnight at room temperature and then neutralized with NaOH, filtered, evaporated in a vacuum at 40° to low volume and finally dried in a vacuum desiccator over H_2SO_4. The residue was a syrup, which was taken up in 30 ml. abs. alcohol; only a very little hygroscopic substance was left undissolved. The alcoholic solution was evaporated to dryness and 1·5 g. of non-hygroscopic substance were obtained. The M.P. was very unsharp, 80–95° (decomp.). Pure fructose melts at 102° or 95°. 0·0997 g. was dissolved to make 10 ml. aqueous solution. In a 2 dm. tube α_D was $-1{\cdot}57°$, and next day had diminished to $-1{\cdot}52°$, i.e. $[\alpha]_D^{20°} = -76{\cdot}2°$. 1·5 g. of the substance yielded 1·05 g. of a diacetone derivative with M.P. 96–97° and $[\alpha]_D^{20°} = -38{\cdot}76°$ (alcohol, $c = 1{\cdot}193$). This polysaccharide also seems therefore to be a laevan.

(3) *Substance from soil* Actinomycete, *strain 47*. [*Ørskov, 1938*]

The bacteria were grown and the substance isolated as in the case of strain 38. 2·0715 g. were heated for 1 hr. on the steam bath with 85 ml. water and 5 ml. *N* HCl. Next day the solution was filtered and made up to 100 ml. The rotation of the solution in a 2 dm. tube was $-3{\cdot}24°$, whence $[\alpha]_D^{20°} = -70{\cdot}4°$.

The solution was neutralized with NaOH and the monosaccharide was isolated as above. Yield *ca.* 2 g. Without purification the material was trans-

formed into a diacetone derivative, of which 1 g. was obtained with M.P. 95·5–96·5 and $[\alpha]_D^{20°} = -38·67$ (alcohol, $c = 1·170$). In this case too fructose seems to be the sole product of hydrolysis.

Comparison with other naturally occurring laevans. Acetylation and methylation

Polylaevans are rather commonly met with in nature. Inulin is the classical example, but in the last few years Schlubach and co-workers [1936; 1937, 1, 2, 3] have isolated a series of fructose anhydrides from different plants, and, which is particularly significant for us, Harrison *et al.* [1930] and Hibbert and co-workers [1930; 1931, 1, 2] have found polylaevans to be synthesized by various micro-organisms when they are grown in sucrose solutions.

The micro-organisms examined by Hibbert *et al.* were *Clostridium gelatigenosum*, *Semiclostridium commune*, *Bacillus laevaniformans*, *B. hemiphloriae*, *Aspergillus sydowi*; the *subtilis* and *mesentericus* groups were specially prone to synthesize polylaevans and the most effective seems to have been *B. mesentericus* Trevisan which can bring about the synthesis from sucrose or raffinose but not from melezitose, lactose, maltose, xylose, glucose or fructose. Ørskov [1938] has found the same for the micro-organisms examined by him.

The polylaevans examined by Schlubach *et al.* were all soluble in water. Those isolated by Hibbert *et al.* were soluble in hot and slightly so in cold water. In most organic solvents they were insoluble, but they dissolved in hot glycerol or ethyleneglycol. These laevans, with acetic anhydride and pyridine, gave triacetates which, after treatment first with methyl sulphate and NaOH and finally with Purdie's reagent, gave trimethyl derivatives. The triacetates and trimethyl derivatives of the different laevans differed in solubility, specific rotation and M.P., the most important difference being that the triacetates of the plant laevans were laevorotatory, whilst those of the bacterial laevans isolated by Hibbert *et al.* were dextrorotatory.

We have therefore prepared the triacetates and trimethyl derivatives of our laevans and have compared their properties with those mentioned above.

(1) *Substance from milk bacteria.* The acetylation was carried out as indicated by Schlubach & Loop [1936]. A triacetate was formed which softened at 90–100° but was completely molten only at 160–170°. (Found: C, 49·73; H, 5·15; CH_3CO, 43·5 %. $(C_{12}H_{16}O_8)_n$ requires C, 50·00; H, 5·60; CH_3CO, 44·9 %.)

The triacetate was nearly insoluble in all solvents. Glacial acetic acid dissolved a small amount when warm, but most of it separated again on cooling; the cooled solution was dextrorotatory, having $\alpha_D + 0·06°$. In chloroform the product had $[\alpha]_D^{20°} + 11·2$, which is to be regarded with reserve as a minimum figure.

The methylation of the triacetate was carried out as indicated by Schlubach & Loop [1936]. The trimethyl derivative was dissolved in benzene and precipitated by light petroleum. A white, non-hygroscopic, amorphous powder was obtained, which softened at 122–125° and was completely molten at 140° $[\alpha]_D^{20°} = -60·9°$ ($CHCl_3$, $c = 2·086$). (Found: OCH_3 44·6 %. $(C_9H_{16}O_5)_n$ requires OCH_3 45·6 %.)

(2) *Substance from soil* Actinomycete, *strain 38.* The triacetate and trimethyl derivative were prepared as mentioned above. The trimethyl derivative softened at 122–125° and was completely molten at 138°. $[\alpha]_D^{20°} = -57·2°$ ($CHCl_3$, $c = 1·958$, $l = 2$). (Found: OCH_3, 46·3 %. Calc. OCH_3, 45·6 %.)

(3) *Substance from soil* Actinomycete, *strain 47.* The trimethyl derivative prepared as above had M.P. 138–140° (softening at 120–125°), $[\alpha]_D^{20°} = -58·8°$ ($CHCl_3$, $c = 1·360$, $l = 2$). On account of lack of material no methoxyl determination was made.

From the properties of the methyl derivatives it appears that all three preparations of polysaccharide are identical. In the table below we have collected the data for different laevans described in the literature.

			Triacetate		Trimethyl derivatives	
	M.P.	$[\alpha]_D$	M.P.	$[\alpha]_D$	M.P.	$[\alpha]_D$
Inulin [Hibbert *et al.*, 1931]	—	−40° water	102–103°, soft 95°	−42·55°, CH_3COOH	138–140°	−50·2°, $CHCl_3$
Laevan [Hibbert *et al.*, 1931]	—	−45·3° water	190°, soft 106°	+21°, $CHCl_3$	145–147°	−87 to −91°, $C_2H_2Cl_4$
Triticin [Schlubach & Peitzner, 1937]	—	−51·4° water	115° or 191°	−15·5°, $CHCl_3$	141–151°	−61·2°, $CHCl_3$
Sinistrin [Schlubach & Loop, 1936]	—	−44°	—	−23·5°, C_6H_6	—	−57°, $CHCl_3$
Asparagosin [Schlubach & Böe, 1937]	197–198° soft 170°	−32·4°	93°, soft 80°	−20·1°, $CHCl_3$	—	−47·8°, $CHCl_3$
Aspholedin [Schlubach & Lendzian, 1937]	—	−30·5°	—	−16·6°	—	—
Own preparations	—	—	160–170°, soft 90°	> +11°, $CHCl_3$	140°, soft 122°	−57 to −60°, $CHCl_3$

The dextrorotation of the triacetate points to the identity of our preparations with the laevans of Hibbert *et al.*, whilst the rotations of the trimethyl derivates resemble more those of the plant laevans examined by Schlubach *et al.* Hibbert *et al.* states that their trimethyllaevans on hydrolysis yield 1:3:4-trimethylfructose, whereas trimethylinulin on hydrolysis yields 3:4:6-trimethylfructose. The amount of trimethyl derivative at our disposal did not permit an examination of the products of hydrolysis, but the investigation is being continued in this direction. We wish to point out, however, that the solubility of our preparations seems to differ both from that of plant laevans and from that of bacterial laevans hitherto described.

Thanks are due to Dr Ørskov, Statens Serum-Institut, Copenhagen, who called my attention to the problem and who has kindly taken the trouble of cultivating the micro-organisms necessary to produce the polysaccharide used in this investigation.

REFERENCES

Ault, Haworth & Hirst (1935). *J. chem. Soc.* p. 1012.
Bridel (1930). *Bull. Soc. chim. biol.* **12**, 921.
Harrison, Tarr & Hibbert (1930). *Canad. J. Res.* **3**, 449.
Hibbert & Tipson (1930). *J. Amer. chem. Soc.* **52**, 2582.
— & Brauns (1931). *Canad. J. Res.* **4**, 596.
— Tipson & Brauns (1931). *Canad. J. Res.* **4**, 221.
Ohle & Koller (1924). *Ber. dtsch. chem. Ges.* **57**, 1366.
Ørskov (1930). *Zbl. Bakt.* I Orig. **119**, 88.
— (1931). *Zbl. Bakt.* I Orig. **120**, 310.
— (1936). *Rep. sec. Int. Congr. Microbiol. London*, 14.
— (1938). *Zbl. Bakt.* II Orig. **98**, 344.
— & Poulsen (1931). *Zbl. Bakt.* I Orig. **120**, 215.
Schlubach & Böe (1937). *Liebigs Ann.* **532**, 191.
— & Lendzian (1937). *Liebigs Ann.* **532**, 200.
— & Loop (1936). *Liebigs Ann.* **523**, 130.
— & Peitzner (1937). *Liebigs Ann.* **530**, 120.

CCLIII. A NEW METHOD FOR THE ISOLATION OF α- AND β-TOCOPHEROLS

By ALBERT RONALD MOSS and JACK CECIL DRUMMOND

From the Department of Biochemistry, University College, London

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Most attempts to isolate the antisterility factor from various oils have made use of saponification as the first stage, thereby effecting a concentration, at least theoretically, of about fourteen times in the case of wheat germ oil. In view of the unexpected sensitivity of the tocopherols to alkali [Moss *et al.* 1938], it seemed to us that a method avoiding the use of the latter would provide information as to the substances responsible for the biological activity of the natural oil, and might also give a better yield. Our experience has been that there are considerable losses during the saponification, even when the treatment is carried out with special precautions.

When it became apparent that the hydroxyl group of the tocopherol molecules was phenolic, it seemed reasonable to think that these compounds might be present in the original oil in the free state although it had usually been assumed, for no very clear reason, that they were esterified. Accordingly, the simple experiment was tried of adsorbing the tocopherols directly from a solution of the original oil. It was entirely successful.

Adsorption of wheat germ oil on Merck's alumina from solution in light petroleum has been found to effect a concentration of the active factor of about fourteen times, both biological and spectroscopic evidence indicating that the fraction recovered from the column contains the whole of the activity of the original oil. The experiments were performed on wheat germ oils from various sources and the results obtained have been uniform both qualitatively and quantitatively.[1]

The chromatogram shows three sets of bands. The first is a pinkish yellow system which moves rapidly down the column. The second is a yellow system which moves slowly downward and divides into upper and lower sections as more material becomes adsorbed; whilst the third is a deep orange yellow band which tends to stay near the top of the column. The tocopherols are associated with the second, slow-moving band system.

Since the great majority of the other constituents of wheat germ oil pass through the alumina column, it was found possible to use much larger amounts of oil for a given size of column than is the usual practice in the adsorption of unsaponifiable material. The maximum amount possible for a $45 \times 4{\cdot}5$ cm. column was found to be 150 g. The limiting factor is the development of the chromatogram toward the bottom of the column, a process occurring more rapidly under the influence of a 10 % solution of oil in light petroleum than with the pure solvent. This circumstance is advantageous in that the usual prolonged washing with large volumes of solvent is unnecessary, the maximum possible

[1] Since this paper was submitted for publication our attention has been drawn by Prof. A. R. Todd to a German patent by the I.G. Farbenindustrie taken out on 30 Jan. 1936 which covers a process of extraction very similar to our own.

separation between the bands occurring during the feeding of the column with the solution. It is then only necessary to wash the column with a small volume of solvent until the filtrate is colourless and practically free from fat, a column of the size mentioned above requiring 300–500 ml.

Eluted separately with mixtures of ether, benzene and methyl alcohol, containing about 80 % of ether, the two yellow bands of the slow-moving system yield yellow oils of which approximately 93 % appears to be triglyceride and esterified sterol. Examined spectroscopically in alcohol, the unsaponifiable materials of these fractions exhibit selective absorption in the ultra-violet, the upper one having a maximum at 295 mμ, and the lower at 292 mμ. Below the lower of the two bands there usually appears, during the washing of the column, a reddish-pink band with which is associated a substance having an ultra-violet absorption with a maximum at 288 mμ.

An attempt was then made to separate the tocopherol from the lower fraction by direct allophanation of the material eluted from the column. Spectroscopic examination before allophanation revealed selective absorption having a rather broad maximum between 286 and 289 mμ. It is reasonable to suppose that this maximum represents the absorption at 292 and 288 mμ shifted toward shorter wave-lengths by the presence of unsaponified fat.

The usual procedure was adopted in the allophanation and after removal of the cyamelide and benzene, three crystalline compounds were isolated from the oil by freezing the solution, first in acetone and then in light petroleum. All three substances were transparent to ultra-violet light and were not further examined. The uncrystallizable residues were adsorbed on alumina from light petroleum, yielding an upper fraction showing absorption with a maximum at 281–283 mμ; and a lower fraction having absorption with a maximum at 277–279 mμ. Readsorption of the two fractions produced no apparent alteration in the physical properties of the mixtures. It is known from the work of John [1937] and of Bergel, Todd & Work [1938] that allophanation causes a shift in the position of the maxima of ultra-violet absorption of 8–9 mμ toward shorter wave-lengths, and it therefore appeared likely that the absorbing substances in these two fractions were the allophanates of compounds having absorption maxima at 288 and 292 mμ; a hypothesis supported by the expected movement of these bands toward the near ultra-violet on alkaline hydrolysis. The hydrolysate of the lower fraction was re-allophanated and the maximum was observed to return to 279 mμ. This substance is being further investigated.

Since the tocopherols of the oily fraction from the original chromatographic absorption seem to be susceptible of allophanation it seems certain either that the hydroxyl groups are free in the original oil, or that they are in combination so labile as to be broken by contact with alumina.

In view of the failure to isolate allophanates by direct treatment of the fraction containing unsaponified fat, it was decided to saponify before allophanation; the comparatively small bulk of the fractions from the column allowed of shorter and more careful treatment during hydrolysis than is possible in handling the larger quantities of the original oil.

It is always found that the upper and lower fractions are each contaminated with a small proportion of the other, so that it is advisable, though not essential, to readsorb the unsaponifiable material before allophanation. This readsorption serves in addition, to remove most of the sterol set free in the hydrolysis; the remainder being precipitated out, as far as possible, with digitonin.

From the material absorbing at 295 mμ, β-tocopherol allophanate was isolated. The orange-coloured oil resulting from the allophanation, dissolved in

light petroleum (B.P. 40–50°), deposited on cooling to 0° a voluminous crystalline precipitate which was free from oil, and which carried with it only a small proportion of colour. Solution of this precipitate in a small volume of cold acetone removed a minute amount of an insoluble flocculent material which was probably a mixture of tritisterol allophanates. The small amount of colour remaining in the acetone-soluble fraction was then removed with blood charcoal from boiling methyl alcoholic solution. The product was recrystallized from anhydrous methyl alcohol, in which it had a very satisfactory temperature coefficient of solubility. At first the substance separated as small, compact, spherical aggregates, but after three recrystallizations as glistening feathery masses of lath-like crystals identical in appearance with those described by Todd *et al.* [1937], M.P. 144·5–145·5°. On anaerobic hydrolysis the allophanate yielded a colourless oil showing, in alcohol or hexane, an ultra-violet absorption with a maximum at 295 mμ $E^{1\%}_{1\,cm.}$ 82 ± 2; persistence 10.

From the lower fraction showing the absorption at 292 mμ, α-tocopherol allophanate was isolated. The flocculent material mentioned above was much more abundant in this fraction, separating along with the allophanate and a good deal of coloured matter from alcoholic solutions. Acetone was employed for the separation; the α-allophanate being moderately soluble and the flocculent material almost entirely insoluble. The same solvent was used for the subsequent recrystallizations, as it was found to keep the oily contaminants and colour in solution in a very satisfactory manner. The final product had M.P. 158·5–159·5°, and gave on hydrolysis a colourless oil which in hexane had selective ultra-violet absorption with the double maximum at 292 mμ and 298 mμ as shown in the curve published by Emerson *et al.* [1937] with $E^{1\%}_{1\,cm.}$ 298 mμ 80 ± 2; persistence 10. In solution in ethyl alcohol the curve showed only a single peak at 292 mμ; $E^{1\%}_{1\,cm.}$ 70 ± 2.

In contrast to the findings of Evans *et al.* [1936] since confirmed by other workers, the α-tocopherol allophanate isolated by the above process was found to show a small optical activity, $\alpha^{25°}_{D} = +4·8$. As it is likely that the α-tocopherol of the original oil is optically active, becoming racemized under the somewhat drastic conditions of the usual saponification, this figure may represent an incomplete racemization under the milder conditions employed for the saponification of our concentrates.

From 2 kg. of wheat germ oil approximately 1 g. of α-tocopherol allophanate and 0·75 g. of β-tocopherol allophanate were isolated.

In a previous paper, from the results of film measurements we postulated the existence in the molecule of a third ring. This was incompatible with the evidence on structure provided by the recent work of Fernholz, Karrer and Todd, from which it seems certain that both α- and β-tocopherols consist essentially of trimethyl-coumaran or -chroman rings bearing hydrocarbon side chains. We have been considerably disturbed by the discrepancy, particularly because the examination of a tocopherol prepared by Dr A. R. Todd and his associates which Danielli recently reported, indicated the area of 30 sq. Å., apparently more compatible with the simpler ring structure.

We were alive to the possibility that some of the preparations previously examined in our laboratory might contain traces of impurities or oxidation products which might give misleading measurements indicating a larger area; and we therefore examined the allophanates and tocopherols described in this paper, having confidence in their purity. To our surprise we obtained values of about 60 sq. Å. for all four compounds. Samples of pure α- and β-tocopherol allophanates, obtained through the kind co-operation of Dr A. R. Todd also

gave measurements indicating areas of about 60 sq. Å. Dr Danielli then very kindly re-examined for us the synthetic compound 5-hydroxy-4:6:7-trimethyl-2-*n*-heptadecylcoumarone for which data had previously been reported by him in a footnote to the publication of Bergel, Jacob *et al.* [1938]. He obtained the value of 51 sq. Å. Hence it appears that the second coumaran or chroman ring is pulled down into the surface, increasing the area to that of a 2-ringed structure. The oxygen atom of these rings must possess a much greater attraction for water than has hitherto been suspected.

Reviewing the foregoing findings we are satisfied now that the film measurements are compatible with the types of formulae recently suggested.

REFERENCES

Bergel, Jacob, Todd & Work (1938). *Nature, Lond.*, **141**, 646.
—— Todd & Work (1938). *J. chem. Soc.* p. 253.
Emerson, Emerson & Evans (1937). *J. biol. Chem.* **122**, 99.
Evans, Emerson & Emerson (1936). *J. biol. Chem.* **113**, 319.
John (1937). *Hoppe-Seyl. Z.* **250**, 11.
Moss, Cuthbertson, Danielli & Drummond (1938). *Chem. and Ind.* 275
Todd, Bergel & Work (1937). *Biochem. J.* **31**, 2257.

CCLIV. THE ISOLATION OF PROGESTERONE AND 3:20-*ALLO*PREGNANOLONE FROM OX ADRENALS

By D. BEALL[1]

British Postgraduate Medical School, London

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THE preparation from the adrenal gland of lipoid extracts which produced oestrous and progestational effects in the uterus of the immature rabbit was reported by Engelhart [1930]. These findings were confirmed by Callow & Parkes [1936], who showed that the active principles could be separated by the method of Allen & Meyer [1933]. They suggested, in view of the structure of the substances already isolated from the adrenal cortex, that the material responsible for the progestational activity might be progesterone or a closely allied compound.

By the courtesy of Prof. T. Reichstein of Zurich a concentrate of ox adrenal gland, obtained from his work on the cortical hormone, was placed at the disposal of Dr A. S. Parkes and made available to the author. Bioassays, carried out by Dr A. S. Parkes, showed this material to have an activity equivalent to 0·5 mg. progesterone per g.

As a preliminary step in the isolation of the active principle the crude material was saponified by treatment with sodium methoxide in anhydrous ether at room temperature. While such a saponification was not necessarily complete it provided a convenient method for the removal of a large amount of inactive acidic material without the destruction of progestational activity.

The alkali-insoluble material was then separated into ketonic and non-ketonic fractions. The ketonic material, on partitioning between suitable solvents, gave a semicrystalline concentrate from which a small amount of 3:20-*allo*-pregnanolone was isolated. Purification of the remainder of the concentrate by alumina adsorption, followed by vacuum sublimation, gave a crystalline product "*X*", M.P. 165–166°, which was subsequently shown to be a complex of *allo*-pregnanolone and progesterone.

Fractionating the crude concentrate has thus resulted in the isolation of progesterone M.P. 121° (uncorr.), $[\alpha]_D^{23°}$ +193°, together with somewhat larger amounts of 3:20-*allo*pregnanolone M.P. 191–192° (uncorr.). Similar results have been obtained by Reichstein [1938] using comparable material and a preliminary report of the work has already appeared [Beall & Reichstein, 1938].

Bioassays carried out throughout the work showed that the activity of the original concentrate could be accounted for by its "*X*" content so that the greater part, if not all of the activity was due to progesterone.

EXPERIMENTAL

Melting points are uncorrected.

Micro-analyses were by Dr A. Schoeller, Berlin.

[1] Beit Memorial Research Fellow.

Preparation of the initial concentrate

The original material was prepared by N. V. Organon, Oss., from 1800 kg. of whole ox adrenals, and the concentrate on partitioning between pentane and 30% methyl alcohol [Reichstein, 1936; 1937] gave a pentane-soluble fraction weighing 1300 g., part of which was used in the present investigation.

Cold saponification

150 ml. of 7·5% sodium methoxide in methyl alcohol were added to 300 g. of the crude pentane-soluble material in 2 l. of dry ether. After 4 hr. at room temperature the mixture was diluted with 1 l. of ether and washed once with 500 ml. of 15% ethyl alcohol, once with 500 ml. of *N* KOH containing 15% ethyl alcohol and then once with 400 ml. of water. The aqueous solutions were combined and extracted with ether, the combined ether extracts being washed and taken to dryness to give 142 g. of "non-sap." Acidification of the alkaline washings, followed by ether extraction, gave 143 g. of "sap." All the progestational activity of the original material was found in the "non-sap." fraction.

Separation of the ketones of the "non-sap."

In a preliminary experiment 9 g. of a "non-sap." fraction in 50 ml. methyl alcohol containing 1·5 ml. of acetic acid were allowed to react for 1·5 hr. with 3 g. of Girard reagent T at room temperature [Reichstein, 1936]. The solution was then poured into 150 g. of ice and water containing 90% of the theoretical amount of NaOH necessary to neutralize the acetic acid, so that the final concentration of alcohol was 25%. It was then extracted four times with ether, the ether being combined and extracted three times with 35 ml. of 25% methyl alcohol.

The aqueous phase was then acidified to Congo red with HCl and, after standing for 1 hr., was ether-extracted to give ketones (A 1) 413 mg. Further acidification of the residual aqueous phase with HCl (10% by volume) and subsequent ether-extraction after 1 hr. gave 32 mg. of additional ketones (A 2). These were combined with the (A 1) fraction to give ketones (A).

The ethereal solution containing the unreacted ketonic and non-ketonic material, after being washed and taken to dryness, was refluxed in 50 ml. methyl alcohol, containing 3 ml. of acetic acid, with 3 g. of Girard reagent T for 1 hr. and then the ketones and non-ketones were separated as before, giving 280 mg. of ketones (B).

Bioassays showed that while the ketones A possessed an activity comparable with that of the original concentrate, the ketones B were inactive when given at five times this dosage level. Therefore in subsequent work the total ketones of the "non-sap." were separated by refluxing with Girard reagent and subsequently fractionated by reaction in the cold with more of this reagent. In this way the 142 g. of "non-sap." from 300 g. of original concentrate gave 4·4 g. of ketones A.

Partition of ketones A

4·4 g. ketones A were dissolved in 140 ml. ethyl alcohol which was then diluted to 200 ml. with water (70% final alcohol conc.) and extracted five times with 100 ml. portions of light petroleum, the latter being extracted in turn three times with 45 ml. portions of 70% alcohol. The alcoholic solutions were combined and extracted five times with 75 ml. portions of benzene which, on being taken to dryness, gave a semicrystalline residue weighing 1·63 g. and containing 92% of active material.

Isolation of 3:20-allopregnanolone and X

The 1·63 g. of semicrystalline benzene-soluble material were dissolved in a minimal amount of ether, several volumes of light petroleum were added and, after standing in the ice box, 303 mg. of solid material were obtained. Crystallization from benzene-light petroleum gave crystals melting at 182–189° which on recrystallization from aqueous ethyl alcohol gave colourless leaflets melting at 191–192° subsequently identified as 3:20-*allo*pregnanolone.

The remainder of the benzene-soluble material (1·3 g.) was dissolved in 60 ml. of alcohol which was then diluted with 90 ml. of water (40% final alcohol conc.) and extracted five times with 100 ml. portions of light petroleum. A small amount of insoluble material was present and was kept in the aqueous phase. The combined light petroleum solutions, which contained all the activity, were taken to dryness and the residue was sublimed *in vacuo*. At 80°/0·05 mm. it gave a small amount of inactive light oil but at 120°/0·05 mm. a partially crystalline yellow sublimate, containing all the active material, was obtained. Attempted crystallization of this sublimate from aqueous alcohol gave 232 mg. of sticky crystals which, on washing with ether, gave 36 mg. of crystals, M.P. 164–165°, which were designated "*X*".

Attempts to obtain more "*X*" from the ether mother liquors by cooling to −80° were unsuccessful, so the residue on evaporation was dissolved in 20 ml. of a mixture of one part benzene and twenty parts light petroleum and run through a column containing 5 g. of active alumina. The column was washed repeatedly with light petroleum until the movement of the coloured zone ceased. The coloured and colourless portions of the column were separated and eluted with a mixture of boiling benzene and alcohol, both extracts being progestationally inactive. The residue from the filtrate, however, had an activity which was equivalent to one-fifth that of progesterone, i.e. was of the same order as that of crystalline "*X*".

In another experiment sublimation of the alumina filtrate (450 mg.) from 3·24 g. of benzene-soluble material, followed by crystallization from aqueous alcohol gave 132 mg. of "*X*", M.P. 162–166°.

Separation of "X" into progesterone and 3:20-allopregnanolone

"*X*", M.P. 164–165°, was recrystallized twice from aqueous alcohol without any change in M.P. Recrystallization of this material from light petroleum gave crystals, M.P. 167–169°, which, on recrystallization from aqueous acetone melted at 165–166°. "*X*" therefore appeared to be a single substance.

Acetylation (pyridine and acetic anhydride at 100°), of 20 mg. "*X*" gave 12 mg. of an acetate, which, recrystallized once from aqueous alcohol, gave crystals melting at 136–137°. These were saponified by refluxing for 15 min. with 10 ml. of 2% alcoholic KOH and on crystallizing from aqueous alcohol formed needles which, alone, or mixed with authentic 3:20:*allo*pregnanolone (M.P. 191–192°) melted at 191–192°.

155 mg. of "*X*" dissolved in 10 ml. of 90% alcohol were treated with a solution of 1·2 g. of digitonin in 10 ml. of 90% alcohol and, after refluxing the mixture for 1 hr. it was left at room temperature overnight and then filtered. The filtrate was concentrated *in vacuo* and ether-extracted, the ether being well washed with dilute acid, alkali and then water. The residue, on evaporation, was sublimed at 120°/0·05 mm. to give 46 mg. of solid. Crystallization of this from light petroleum gave 10 mg. of progesterone which had M.P. 121° alone, and

mixed with authentic progesterone (M.P. 127°) melted at 121–123°. $[\alpha]_D^{22°}$ +193° (l = 0·5, c = 1·01% in ethyl alcohol). Its activity, on bioassay, was similar to that of progesterone. (Found: C, 80·1; H, 9·64%. $C_{21}H_{30}O_2$ requires C, 80·2; H, 9·62%.)

Identification of 3:20-allopregnanolone

76 mg. of crude *allo*pregnanolone, M.P. 178–185°, were acetylated and the acetate (65 mg.) was recrystallized twice from aqueous alcohol to give 52 mg. of *allo*pregnanolone acetate (leaflets) which alone, or mixed with authentic *allo*pregnanolone acetate (M.P. 142–143°), melted at 142–143°.

Saponification of the acetate in ether with sodium methoxide for 1 hr. followed by recrystallization from aqueous alcohol gave 32 mg. of leaflets which, alone or mixed with an authentic specimen of *allo*pregnanolone (M.P. 191–192°), melted at 191–192°. (Found: C, 79·1; 79·1; H, 10·5; 10·5%. $C_{21}H_{34}O_2$ requires C, 79·2; H, 10·8%.)

30 mg. of this pure *allo*pregnanolone were dissolved in 2 ml. of 90% acetic acid and treated with a solution of 50 mg. of chromic acid in 2 ml. of 90% acetic acid [cf. Butenandt *et al.* 1934]. After 6 hr. at room temperature the mixture was diluted with water, filtered, washed and the precipitate, recrystallized from aqueous alcohol, gave 12 mg. of *allo*pregnandione (leaflets), which, alone or mixed with authentic *allo*pregnandione (M.P. 198–199°), melted at 198–199°.

The author wishes to thank Prof. T. Reichstein of Zurich for supplying the ox adrenal concentrate, for specimens of 3:20-*allo*pregnanolone and its acetate and in particular for his cordial exchange of information during the course of the work. The author is also deeply indebted to Dr A. S. Parkes, National Institute for Medical Research, London, without whose co-operation with the biological assays it would have been impossible to have carried out the investigation. Acknowledgment is also made to the Medical Research Council for a grant towards the partial cost of the work.

REFERENCES

Allen & Meyer (1933). *Amer. J. Physiol.* **106**, 55.
Beall & Reichstein (1938). *Nature, Lond.*, **142**, 479.
Butenandt, Westphal & Hohlweg (1934). *Hoppe-Seyl. Z.* **227**, 84.
Callow & Parkes (1936). *J. Physiol.* **87**, 28 *P*.
Engelhart (1930). *Klin. Wschr.* **9**, 2114.
Reichstein (1936). *Helv. Chim. Acta*, **19**, 1107.
—— (1937). *Helv. Chim. Acta*, **20**, 953.
—— (1938). *Helv. Chim. Acta*, **21** (in the Press).

CCLV. ACTION OF ARSENATE IN GLYCOLYSIS

By RAMAN KOCHUKRISHNA PILLAI

From the Biochemical Laboratory, Cambridge

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The activating effect of arsenate on glycolysis in alcoholic fermentation as well as in muscle brei and extracts has been known for a long time [Harden & Young, 1906; 1911; Meyerhof, 1921], and it was also known that this activation is due specifically to increased hexosediphosphate breakdown [Harden & Young, 1911; Meyerhof, 1918]. The assumption that the effect of the arsenate was to activate a hexosediphosphatase splitting hexosediphosphate into hexose and inorganic phosphate was early abandoned [Meyerhof, 1927; Raymond, 1928; Macfarlane, 1930; Harden, 1932] as it was shown that arsenate activation of hexosediphosphate breakdown could not be obtained in the presence of the phosphatase only, independently of increased fermentation. Shortly after, it was found in yeast juice as well as in muscle extract that the production of alcohol or lactic acid from dihydroxyacetonephosphate, the breakdown product of hexosediphosphate, was increased by arsenate as much as that from hexosediphosphate, and further that the oxidoreduction of triosephosphate and acetaldehyde or pyruvate as well as the dephosphorylation of phosphoglycerate (phosphopyruvate) proceeded very much faster in the presence of arsenate [Meyerhof, 1934; Meyerhof & Kiessling, 1935]. Several attempts were made to explain the increased dephosphorylation of phosphoglycerate in the presence of arsenate. Since phosphoglycerate was known to be dephosphorylated after conversion into phosphopyruvate by transfer of its phosphate to adenylic acid forming adenylpyrophosphate and pyruvate, it was thought that arsenate, by activating the enzyme adenylpyrophosphatase, might increase the production of adenylic acid from the pyrophosphate. However, Pett & Wynne [1934] and Schäffner & Krumey [1936] showed conclusively that all the phosphatases investigated with the possible exception of adenylpyrophosphatase were not activated but even inhibited by arsenate; Needham & Pillai [1937] later showed that with adenylpyrophosphatase also the activation was only very slight and could not in any case account for such very greatly increased production of phosphate as is usually observed with arsenate. The dephosphorylation of phosphoglycerate in the presence of arsenate and catalytic amounts of adenylic acid in yeast juice or muscle extract occurs as fast as the transfer of phosphate from phosphoglycerate to equivalent amounts of adenylic acid, and it was therefore supposed that in such extracts the adenylic acid is somehow constantly being regenerated, but Meyerhof & Kiessling [1936] could not find any evidence of this and postulated the formation and breakdown of a cozymasepyrophosphate.

The coupled esterification of adenylic acid with inorganic phosphate consequent upon the oxidation of triosephosphate in muscle extract had by now been demonstrated [Meyerhof *et al.* 1937, 2; Needham & Pillai, 1937]. Needham & Pillai found that if arsenate was present during the oxidation no accumulation of adenylpyrophosphate resulted and they concluded that arsenate inhibited the coupling of esterification and oxidation. This interpretation was advanced

by other workers also [Meyerhof, 1937; Meyerhof *et al.* 1937, 1; 1938, 1] who supposed that the rapid oxidation of triosephosphate could take place only in conjunction with the esterification of inorganic phosphate with adenylic acid, and that arsenate, by inhibiting the coupling of oxidation and esterification, enabled the former to go on at the same rate even in the absence of large amounts of adenylic acid and inorganic phosphate. This assumption, besides explaining the observed activation by arsenate of the reaction triosephosphate + pyruvate (or acetaldehyde), would also show why hexosediphosphate breakdown itself can be influenced by arsenate in still another way. In normal glycolysis, where glycogen or glucose is the substrate, the adenylpyrophosphate formed during the dephosphorylation of phosphopyruvate is immediately dephosphorylated by donating its phosphate to glycogen or glucose, quite apart from the action of the adenylpyrophosphatase which is considered to be much slower. The adenylic acid is thus continuously regenerated to dephosphorylate more and more phosphopyruvate. When hexosediphosphate is the substrate it cannot however serve as phosphate acceptor for adenylpyrophosphate. The coupled esterification of adenylic acid during oxidation of triosephosphate goes on in both cases, but when arsenate is present the adenylic acid is not esterified and is free to dephosphorylate phosphopyruvate so that the breakdown of hexosediphosphate proceeds readily. In the case of glucose or glycogen this mechanism is of less importance.

Shortly afterwards Pillai [1938] showed that phosphoglycerate could be rapidly dephosphorylated in long-dialysed extracts of acetone muscle powder in the presence of arsenate if catalytic amounts of adenylic acid, Mg and cozymase are present, a result somewhat different from that of Meyerhof *et al.* [1937, 1] who found that in their A and B protein enzyme system a trace of hexosediphosphate too was necessary. They also stated that if an excess of adenylpyrophosphate is added to the arsenate-activated system, the phosphopyruvate breaks down but not the adenylpyrophosphate.

Since the arsenate activates not only the breakdown of hexosediphosphate as a whole but also all the known intermediate reactions individually, it seemed possible that the inhibition of the coupled esterification observed might be more apparent than real and that the action of the arsenate on this reaction should be investigated more fully. The muscle extracts used for the dephosphorylation experiments with phosphoglycerate contained no adenylpyrophosphatase [Pillai, 1938], and since, besides Mg and cozymase, adenylic acid was indispensable, it seemed, assuming as usual that the phosphopyruvate was dephosphorylated by transferring phosphate to adenylic acid, obvious to conclude that the adenylpyrophosphate must constantly be breaking down and reforming in the presence of arsenate to enable this reaction to take place. The effect of the arsenate in inhibiting the coupling might therefore very well be due to the accelerated breakdown by some mechanism, other than adenylpyrophosphatase, of the adenylpyrophosphate that is formed. The experiments described below show that in fact this is quite probable and that in the presence of arsenate the adenylpyrophosphate readily breaks down if a trace of phosphoglycerate, fluoride and cozymase are added.

Experimental

Enzyme preparation

An aqueous extract of a muscle powder prepared by precipitating fresh rabbit muscle extract with acetone was used [Needham & Pillai, 1937]. The extract was dialysed in cellophane tubes for 1–4 days according to the needs of the experiment. The enzyme solution does not contain any adenylpyrophosphatase.

Chemical preparations. Besides the preparations described by Pillai [1938], synthetic phosphopyruvate was made by the method of Kiessling [1936].

Methods of estimation. The usual methods adopted were the same as those given before [Pillai, 1938]. Adenylic acid and adenylpyrophosphate were determined when necessary by the deaminase method of Parnas & Lutwak-Mann [1935]. Details of the procedure will be found elsewhere [Needham & Pillai, 1937].

Experimental procedure. For most of the experiments 2 ml. enzyme solution were diluted to 4–6 ml. with the several additions of substrates, coenzymes, fluoride, arsenate etc. The amounts of these additions are given along with the experimental results. 0·3 ml. 6% $NaHCO_3$ was used as buffer in most cases and the experiments were carried out in ordinary 25 ml. centrifuge tubes. Suitable controls were always done simultaneously. The time of experiment was 15–30 min. at 38° or at room temp. (20°). Trichloroacetic acid was used to precipitate the proteins and the P estimations were carried out after filtration through dry filter paper. In the creatinephosphate estimations centrifuging was found quicker and more satisfactory than filtration.

The results are expressed, unless otherwise stated, as mg. increase or decrease per 2 ml. extract.

Details of experiments

It has been suggested that arsenate increases the rate of oxidoreduction between triosephosphate and pyruvate because this reaction goes on rapidly only when accompanied by simultaneous esterification of adenylic acid, and arsenate, by breaking this coupling, allows the reaction to proceed independently of the esterification [Meyerhof *et al.* 1937, 1; 1938, 1]. The following table shows the yields of lactic acid obtained during the dismutation of triosephosphate +pyruvate in the presence and absence of adenylic acid and inorganic P. The enzyme solution was an extract dialysed 73 hr. and containing practically no inorganic P.

Exp. I. All the tubes, including the control, received 2 ml. enzyme solution, 0·6 ml. *M*/6 Na hexosediphosphate, 0·8 ml. *M*/5 Na pyruvate, 0·6 ml. 0·15% cozymase, 0·1 ml. 2·5% $MgCl_2, 6H_2O$, 0·15 ml. 0·4 *M* NaF and 0·3 ml. 6% $NaHCO_3$.

	mg. lactic acid
Control	+2·2
+phosphate (1 ml. 0·16 *M*)	+4·5
+phosphate+adenylic acid (12 mg.)	+6·6

The adenylic acid was neutralized before addition and all tubes made up to 6 ml. with water. Time of exp. 15 min. at 38°.

Both inorganic P and adenylic acid give increased dismutation but even in their absence there is a fairly active reaction. The effect of adding P alone seemed peculiar, but the following exp. shows that even in the absence of adenylic acid esterification is taking place, though of a different nature.

Exp. II. All preliminary additions as in Exp. I, except that an extract dialysed only 25 hr. and therefore probably containing traces of adenylic acid was used. 30 min. at 38° C.

	mg. inorganic P
Control+phosphate (1 ml. 0·16 *M*)	−1·01
+phosphate+arsenate (0·15 ml. *M*/10)	+0·22

There is a considerable disappearance of inorganic phosphate when it is present during dismutation even in the absence of adenylic acid; this is inhibited by arsenate. The ester formed cannot be adenylpyrophosphate or cozymase-pyrophosphate as the amount of adenylic acid present in the 25 hr.-dialysed

extract must be very small, also only 0·15 mg. cozymase is added (0·5 ml. 0·15% solution of 20% pure cozymase). The ester was not identified but it might be formed by esterification of some of the pyruvate, the resulting phosphopyruvate being dephosphorylated in the presence of arsenate and the traces of adenylic acid still present. In some later experiments where an extract dialysed for much longer was used the esterification was found to be less but still noticeable.

The above experiment seems to show that the effect of the arsenate might be to increase the breakdown of any ester formed rather than to inhibit the esterification itself since it is known that arsenate activates the breakdown of phosphopyruvate. The next experiment shows that this is very probably the case for the oxidoreduction of triosephosphate + pyruvate and the corresponding esterification of adenylic acid.

Exp. III. Besides the preliminary additions given in Exp. I all tubes received 12 mg. adenylic acid and 1 ml. 0·16 *M* phosphate. In no. 3 the arsenate was added at the beginning of the experiment, but in 4 only after 15 min. at 38° when it was kept at 38° for a further 15 min.

	mg. inorganic P		min. at 38°
1. Control	−2·08	−2·16	15
2. Control	—	−2·37	30
3. + arsenate (0·15 ml. *M*/10)	−0·07	+0·13	15
4. + arsenate	−0·29	−0·31	15 + 15

The effect of the arsenate added before or after esterification is the same. In no. 4 the esterification has taken place but arsenate added later breaks down all the adenylpyrophosphate formed so that there is no difference between nos. 3 and 4. Whether in the presence of arsenate the esterification takes place at all is impossible of direct proof.

In order to see how the activation of phosphate breakdown actually happens it was necessary to exclude once for all the possibility of arsenate activation of hexosediphosphatase, to make sure that the extra inorganic P in the presence of arsenate did not come from this source. Exp. IV shows that in the presence of NaF only, the arsenate considerably inhibits the production of inorganic P from hexosediphosphate. This however is due to the fact that the arsenate greatly activates the dismutation of triosephosphate and thus reduces the effective concentration of hexosediphosphate itself. With both NaF and iodoacetate the inhibition is very much less but still noticeable.

Exp. IV. All tubes received 2 ml. extract, 0·6 ml. *M*/6 hexosediphosphate 0·1 ml. 2·5% $MgCl_2$, 0·1 ml. of 0·4 *M* NaF and 0·3 ml. 6% $NaHCO_3$. Made up to 4 ml. with additions. All 30 min. at 38°.

	mg. inorganic P		
Control	0·91	—	0·69
+ arsenate (0·1 ml. *M*/10)	0·37	—	0·45
+ iodoacetate (0·1 ml. *M*/10)	—	0·79	0·72
+ iodoacetate + arsenate	—	0·66	0·63

The next point was to test whether added adenylpyrophosphate would be broken down during the dismutation of hexosediphosphate + pyruvate in the presence of arsenate and the following experiment shows that this is indeed the case.

Exp. V. All preliminary additions as in Exp. I. 15 min. at 38°.

	mg. inorganic P
Control	−0·04
+ A.T.P. (0·8 mg. pyro-P)	+0·01
+ arsenate	+0·33
+ arsenate + A.T.P.	+1·19

In the absence of arsenate the adenylpyrophosphate does not break down.

It seemed therefore as if the oxidoreduction itself or one of the products formed could, in the presence of arsenate, cause adenylpyrophosphate to split off inorganic P. In accordance with this assumption the dismutation of two triosephosphate molecules alone without pyruvate could bring about decomposition of A.T.P., as is shown in the following experiment which was made with all additions as in Exp. V except for pyruvate which was omitted:

	mg. inorganic P
Control + arsenate	0·28
+ arsenate + A.T.P.	0·72

Since for the dephosphorylation of phosphopyruvate in muscle extract in the presence of arsenate and no adenylpyrophosphatase a small amount of adenylic acid acts catalytically and is indispensable, there is an obvious possibility that adenylpyrophosphate is continually being broken down and reformed. One would therefore expect that large amounts of adenylpyrophosphate should break down in extracts in which phosphopyruvate is at the same time experiencing decomposition. Addition of considerable amounts of adenylpyrophosphate to muscle extracts in which phosphoglycerate was being dephosphorylated in the presence of arsenate, however, failed to produce appreciable extra dephosphorylation.

Exp. VI. 0·5 ml. 0·1 *M* phosphoglycerate, 0·5 ml. 0·15% cozymase, 0·1 ml. $MgCl_2$, 0·2 ml. A.T.P. (0·1 mg. pyro-P) and 0·25 *M*/50 arsenate were added to 2 ml. muscle extract (dialysed 23 hr.) in the control and the other tubes. Made up to 5 ml. with 0·3 ml. $NaHCO_3$, water and other additions if any.

	Inorganic P	min. at 38°
	1·54	30
Control	1·60	60
+ A.T.P. (0·6 mg. pyro-P)	1·68	30

The negative result in this case, however, might be due to the fact that we are here dealing with the same enzyme reaction in both cases (the dephosphorylation of A.T.P. and phosphoglycerate requiring the same enzyme), or because at the moment when all phosphoglycerate has been dephosphorylated the A.T.P. has still remained intact. The former is unlikely because practically all the phosphoglycerate has broken down in 30 min. The latter would follow from the fact that so long as any phosphoglycerate (phosphopyruvate) is present the adenylic acid formed by breakdown of A.T.P. would be immediately rephosphorylated. Addition of NaF would of course prevent the dephosphorylation of phosphoglycerate and the consequent regeneration of A.T.P. The following experiment in which this was done shows that under these conditions the adenylpyrophosphate is completely broken down [compare Meyerhof *et al.* 1937, 1].

Exp. VII. All additions as in Exp. VI except that 0·5 ml. 0·4 *M* NaF was added (conc. of NaF *M*/28).

	Inorganic P
Control	0·30
+ A.T.P. (0·6 mg. pyro-P)	0·86

That the adenylpyrophosphate has actually broken down and has not merely removed the inhibition of phosphoglycerate dephosphorylation by NaF [Runnström & Hemberg, 1937] was shown by precipitating the residual adenylpyrophosphate with Ba acetate and estimating NH_2-N by the method of Parnas & Lutwak-Mann [1935]. There was no A.T.P. left. Further proof of this is given

below in experiments with only a trace of phosphoglycerate, which could not provide more than a fraction of the inorganic P by its own decomposition.

It seemed therefore that in the mere presence of phosphoglycerate and the complete oxidoreduction enzymes with arsenate adenylpyrophosphate could break down. Obviously this reaction is at the basis of the dephosphorylation of phosphoglycerate observed [Pillai, 1938] in the same system. There a small amount of adenylpyrophosphate is able to act catalytically, for immediately on decomposition it is rephosphorylated by some of the phosphopyruvate and this would go on continuously till all the phosphoglycerate has been decomposed. In view of the fact that the mechanism of the coupled esterification of adenylic acid

triosephosphate + cozymase + adenylic acid + inorganic phosphate
→ phosphoglycerate + reduced cozymase + A.T.P., ...(A)

has been shown to be reversible [Meyerhof *et al.* 1938, 2]

A.T.P. + phosphoglycerate + reduced cozymase
→ triosephosphate + cozymase + adenylic acid + P, ...(B)

it was natural to suppose that the dephosphorylation of phosphoglycerate in the presence of traces of cozymase and A.T.P. was related to reaction B. This reaction is stoichiometric, but in the presence of alcohol or lactic acid to reduce the cozymase as soon as it is formed a small amount of cozymase can act catalytically. Leaving aside for a moment the mechanism of cozymase function one would suppose that part of the phosphoglycerate would undergo conversion into triosephosphate during its dephosphorylation. No accumulation of triosephosphate can however be expected, since triosephosphate in the presence of traces of cozymase and of the pyruvate formed by the dephosphorylation of phosphopyruvate would be reoxidized completely to phosphoglycerate when arsenate is present in the system [Adler & Günther, 1938]. As a matter of fact neither triosephosphate nor lactic acid could be detected among the products formed. However, if conversion into triosephosphate is concerned at all in the reaction one should get an inhibition of the dephosphorylation of phosphoglycerate if this change is prevented. By using synthetic phosphopyruvate instead of phosphoglycerate and employing NaF to prevent any conversion of the former into phosphoglycerate and then into triosephosphate, the dephosphorylation of phosphopyruvate should be considerably inhibited if the supposition is true. The following experiment in which this was done shows that the dephosphorylation of phosphopyruvate in the presence of traces of adenylic acid and cozymase is inhibited by NaF only slightly, both in presence as well as absence of arsenate.

Exp. VIII. 1·2 ml. Na phosphopyruvate containing 2·7 mg. phosphopyruvic P, 0·2 ml. A.T.P. containing 0·1 mg. pyro-P, 0·5 ml. 0·2% cozymase, 0·1 ml. 2·5% $MgCl_2$, $6H_2O$ and 0·3 ml. 6% $NaHCO_3$ were added to all tubes together with 2 ml. extract dialysed 23 hr. Made up to 5 ml. with further additions or water.

	Inorganic P	
Control	0·55	30 min. at 38°
+ NaF (0·3 ml. 0·4 *M*)	0·39	
+ arsenate (0·25 ml. *M*/50)	2·01	15 min. at 20°
+ arsenate + NaF	1·58	

The slight inhibition observed may be due to injury to the enzymes by the high concentration of NaF (*M*/40), or because, since only a trace of phospho-

glycerate is required for the reaction and the NaF poisoning is not 100% effective, especially with large amounts of added phosphopyruvate, a small but sufficient amount of phosphoglycerate has formed in a short time. The following experiment in which decreasing amounts of phosphoglycerate are added in the presence of NaF shows that even with a trace of phosphoglycerate (0·12 mg. P) the adenylpyrophosphate is completely dephosphorylated.

Exp. IX. 0·5 ml. 0·2% cozymase, 0·1 ml. $MgCl_2$, 0·3 ml. *M*/50 arsenate, 0·6 ml. 0·4 *M* NaF and 0·3 ml. 6% $NaHCO_3$ were added to 2 ml. extract (dialysed 23 hr.) in all tubes including controls. Made up to 6 ml. with water or further additions. In each case a control containing the same amount of phosphoglycerate and a trace of A.T.P. was run to compensate for any dephosphorylation of the phosphoglycerate itself. 30 min. at 38°.

	mg. inorganic P
Control + 0·5 ml. *M*/5 phosphoglycerate + A.T.P. (0·75 mg. pyro-P)	−0·66
+ 0·1 ml. *M*/5 ,, + A.T.P. (0·75 mg. pyro-P)	0·60
+ 0·5 ml. *M*/50 ,, + A.T.P. (0·83 mg. pyro-P)	0·87
+ 0·2 ml. *M*/50 ,, + A.T.P. (0·83 mg. pyro-P)	0·79

Practically all the pyrophosphate is broken down under these conditions and even at room temp. the reaction is quite rapid.

Exp. X. All additions were the same as in previous experiment except that 0·2 ml. *M*/50 phosphoglycerate and A.T.P. (1·1 mg. pyro-P) were added to all tubes. Reaction at room temp. (20°).

	Inorganic P
15 min.	0·69
30 ,,	0·84
60 ,,	0·99

It was observed that Mg is not necessary, but cozymase is indispensable and that iodoacetate completely inhibits the reaction. In the absence of NaF the dephosphorylation is very small, evidently owing to the disappearance of the phosphoglycerate by dephosphorylation after conversion into phosphopyruvate.

Exp. XI. All tubes received 2 ml. extract dialysed 77 hr., 0·2 ml. *M*/50 phosphoglycerate, 2 ml. A.T.P. containing 1·1 mg. pyro-P, 3 ml. *M*/50 arsenate and 0·3 ml. 6% $NaHCO_3$. Made up to 6 ml. with further additions or water. 0·5 ml. 0·2% cozymase, 0·1 ml. 2·5% $MgCl_2$, $6H_2O$, 0·6 ml. 0·4 *M* NaF and 0·1 ml. *M*/10 iodoacetate added as indicated. 15 min. at room temp.

	Inorganic P
Control + NaF + Mg + cozymase	0·69
+ NaF + Mg only	0·46
+ NaF + cozymase only	0·68
+ cozymase + Mg (no NaF)	0·22
+ cozymase + Mg + NaF + iodoacetate	0·08

The dephosphorylation is quite high without cozymase but this is due to the fact that the adenylpyrophosphate used contains small amounts of cozymase as impurity. With adenylpyrophosphate freed from cozymase by leaving the solution overnight at *p*H 9·0, at which alkalinity the cozymase is inactivated, the indispensability of cozymase is quite clear. 15 min. at room temp.

	Inorganic P
No cozymase	0·07
+ cozymase	0·21

That no impurity in the sodium phosphoglycerate itself is responsible for the activation of adenylpyrophosphate breakdown can be shown by adding a small amount of synthetic phosphopyruvate to the extract, allowing it to remain

a few min. so that some of it is converted into phosphoglycerate and then adding the NaF, arsenate, coenzymes and A.T.P. This works as satisfactorily as the phosphoglycerate itself.

Sodium glycerate substituted for phosphoglycerate is completely inactive so that the possibility of decomposition of phosphoglycerate in such manner in the dephosphorylation system may be set aside. α-glycerophosphate and pyruvate have little effect. Even glyceraldehyde and pyruvate together did not influence the dephosphorylation of A.T.P. to any considerable extent beyond that by pyruvate itself.

Exp. XII. 2 ml. extract, 0·5 ml. cozymase, 0·1 ml. $MgCl_2$, 0·3 ml. *M*/50 arsenate, 1·7 ml. A.T.P. containing 1 mg. pyro-P and 0·3 ml. $NaHCO_3$ were mixed and made up to 6 ml. with water or further additions as follows: 0·4 ml. *M*/5 Na glycerate, 0·5 ml. *M*/5·5 Na glycerophosphate, 0·5 ml. *M*/5 pyruvate, 0·5 *M*/5 glyceraldehyde and 0·6 ml. 0·4 *M* NaF. Time 30 min.

	Inorganic P	Temp.
Control + Na glycerate + NaF	+0·01	20°
+ glycerophosphate	+0·07	38°
+ glycerophosphate + NaF	+0·02	38°
+ pyruvate + NaF	+0·18	38°
+ pyruvate + glyceraldehyde	+0·31	38°

The reaction would therefore seem to be specific for phosphoglycerate.

In the absence of arsenate, A.T.P. is not decomposed in this enzyme system even in the presence of large amounts of phosphoglycerate and NaF, notwithstanding the fact that if no NaF is added phosphoglycerate itself will be dephosphorylated in such an extract with small amounts of A.T.P. added as coenzyme.

It is of interest to see whether creatinephosphate will be decomposed under the same conditions as adenylpyrophosphate in this system. The following experiment shows that in the presence of traces of phosphoglycerate and arsenate creatinephosphate will split off inorganic P even in the absence of adenylic acid. Mg is necessary for this, unlike the decomposition of A.T.P., and iodoacetate inhibits the reaction. Cozymase is indispensable.

Exp. XIII. 2 ml. extract dialysed 94 hr., 2 ml. creatinephosphate containing 1·2 mg. P, 0·2 ml. *M*/50 phosphoglycerate, 0·6 ml. 0·4 *M* NaF, 0·3 ml. *M*/50 arsenate, and 0·3 ml. 6% $NaHCO_3$ were mixed. Made up to about 6 ml. with further additions as indicated. 0·5 ml. 0·15% cozymase, 0·2 ml. 4% adenylic acid and 0·1 ml. 2·5% $MgCl_2$, $6H_2O$. 30 min. at 20°.

	Inorganic P		
Control + Mg	0·22	—	—
+ Mg + cozymase	0·63	0·76	0·88
+ Mg + cozymase + adenylic acid	0·68	—	—
+ cozymase	—	—	0·49
+ cozymase + Mg + iodoacetate	—	—	0·10

It is curious that adenylic acid is unnecessary for the dephosphorylation of creatinephosphate. That the creatinephosphate itself contains no trace of adenylic acid as impurity can be shown by estimating the adenylic acid by the deaminase method of Parnas and also indirectly. It is known that adenylic acid is obligatory for the dephosphorylation of phosphoglycerate in the presence of arsenate and of the complete oxidoreduction enzymes [Pillai, 1938]. Creatinephosphate is also broken down in such an extract in the presence of phosphoglycerate. Even supposing that the mechanism of breakdown is the same in

both cases, if a small amount of creatinephosphate, which will be completely decomposed, is added to phosphoglycerate in the same extract with all additions except adenylic acid, one would expect some of the phosphoglycerate also to be dephosphorylated provided that the creatinephosphate contains some adenylic acid as impurity, but not otherwise. The following experiment shows that no extra phosphoglycerate is broken down if creatinephosphate alone is added, but the further addition of a trace of adenylic acid (0·1 mg.) immediately increases the production of inorganic P.

Exp. XIV. To 2 ml. extract dialysed 94 hr., 0·5 ml. *M*/5 phosphoglycerate, 0·5 ml. 0·15% cozymase, 0·1 ml. 2·5% $MgCl_2$, $6H_2O$, 0·25 ml. *M*/50 arsenate and 0·3 ml. 6% $NaHCO_3$ were added and made up to 5 ml. with water or further additions as follows: creatinephosphate (containing 0·6 mg. P) and 0·1 mg. adenylic acid when indicated. 30 min. at room temp.

	Inorganic P
Control	0·38
+ NaF	0·17
+ NaF + creatinephosphate	0·77
+ creatinephosphate	0·90
+ creatinephosphate + adenylic acid (0·1 mg.)	1·37

Further, adenylic acid does not activate the production of phosphate from creatinephosphate alone.

	Inorganic P
Creatinephosphate only (1·2 mg. P)	0·63
+ adenylic acid (0·8 mg.)	0·68

Creatinephosphate therefore seems to be able to split off inorganic phosphate in the presence of arsenate without the agency of adenylic acid or at most in the presence of extremely minute amounts.

It has already been mentioned that the mechanism of breakdown of A.T.P. in the presence of phosphoglycerate might possibly be related to the reaction phosphoglycerate + reduced cozymase + A.T.P. → triosephosphate + oxidized cozymase + adenylic acid + P which has been shown to occur in muscle extracts. This requires reduced cozymase to start the reaction and since neither reduced cozymase nor any reducing agent such as lactic acid or acetaldehyde was added, it was thought possible that some material present in the extract itself might bring about this reduction.

Experiments in which considerable amounts of cozymase were added to the extract, either alone or in the presence of arsenate or with all further additions, failed to show the presence of any reduced cozymase when examined spectrophotometrically. Reduced coenzyme if present at all must be in very small amounts as the method detects amounts as low as 0·05 mg. reduced cozymase [Warburg *et al.* 1935].

Discussion

The breakdown of adenylpyrophosphate by muscle extract containing no adenylpyrophosphatase, in the presence of arsenate, cozymase and small traces of phosphoglycerate (prevented from decomposition by NaF), has been demonstrated. This reaction is the basis of the dephosphorylation of phosphoglycerate in the presence of catalytic amounts of adenylic acid, observed under the same conditions in muscle extract [Pillai, 1938]. There the adenylic acid is esterified by phosphopyruvate and broken down continuously by the excess phosphoglycerate, and the reaction requires the presence of Mg, though adenylpyrophosphate itself seems to break down in its absence. Since Mg is obligatory for

the transfer of phosphate from phosphopyruvate to adenylic acid, it is easy to see that the dephosphorylation of phosphopyruvate proceeds via adenylic acid.

In the absence of arsenate, adenylpyrophosphate does not decompose even in the presence of very large amounts of phosphoglycerate and NaF, though phosphoglycerate itself under these conditions undergoes considerable dephosphorylation in the absence of NaF if a trace of adenylpyrophosphate is added. This shows that the mechanism of the reaction in which, in the absence of arsenate, phosphoglycerate is dephosphorylated in muscle extract containing no adenylpyrophosphatase and only traces of A.T.P. must be something entirely different from the reaction in the presence of arsenate as has been noticed before [Pillai, 1938].

It is difficult to understand the mechanism of the reaction by which adenylpyrophosphate is decomposed by muscle extract in the presence of traces of phosphoglycerate, cozymase and arsenate. It has been shown that the reaction

$$\text{triosephosphate} + \text{cozymase} + \text{adenylic acid} + \text{phosphate} \rightleftharpoons \text{phosphoglycerate} + \text{reduced cozymase} + \text{A.T.P.}$$

is reversible and can take place both in yeast and muscle extracts. The reaction is stoichiometric and the function of the A.T.P. in the reaction from right to left is to provide the energy by its breakdown to adenylic acid and inorganic phosphate for the conversion of phosphoglycerate into triosephosphate, an endothermic reaction. If lactic acid or alcohol is added to reduce the cozymase as soon as it is oxidized by the phosphoglycerate a small amount of cozymase can act catalytically. A small amount of A.T.P. would also suffice if some phosphate donator like creatinephosphate were added or the NaF omitted, so that some of the phosphopyruvate might itself rephosphorylate adenylic acid.

In the presence of arsenate not only does a trace of cozymase suffice but even a small amount of phosphoglycerate can act catalytically provided that NaF is added to prevent its decomposition to phosphate and pyruvic acid. It has been shown that the phosphoglycerate cannot act by directly splitting off phosphate since Na glycerate is inactive; also the inactivation by iodoacetate shows that the reaction involves some kind of oxidoreduction. Arguing from analogy with phosphoglycerate, glycerophosphate should be capable of reducing cozymase and being oxidized to triosephosphate in the same enzyme system but it has very little action upon the dephosphorylation of A.T.P. Similarly pyruvate, as well as pyruvate + glyceraldehyde, which can form a powerful oxidoreduction system with the formation of lactic acid, has comparatively little action. This breakdown of A.T.P. in the presence of arsenate, like the coupled esterification of adenylic acid, therefore seems to be more or less specific to phosphoglycerate $\rightleftharpoons$ triosephosphate.

It will be observed that no reduced coenzyme could be found spectrophotometrically in the enzyme system producing dephosphorylation of A.T.P. even when large amounts of cozymase were added. The possibility of small amounts of reduced cozymase which cannot be detected by the analytical method employed being present must however be borne in mind.

That cozymase can act under certain circumstances as phosphate carrier must now be considered to have been proved beyond doubt [see Ohlmeyer & Ochoa, 1937] and it is likely that in all the reactions considered below a cozymase-pyrophosphate is an intermediate compound, both in esterification and in liberation of inorganic phosphate from A.T.P. or creatinephosphate. The function of the adenylic acid, arguing from analogy with creatine, would then be to act as an acceptor of phosphate from the cozymasepyrophosphate, and of the A.T.P., like creatinephosphate, to be a donator of phosphate to cozymase.

Suppose now that a small amount of reduced cozymase is initially present to start the reaction from right to left in the reversible coupled reaction given above. It will be seen that very soon an equilibrium will be established, when as much triosephosphate will be oxidized by the cozymase to phosphoglycerate as phosphoglycerate is being reduced by the reduced coenzyme. No adenylpyrophosphate will however disappear in the sequel because an equivalent amount of inorganic phosphate will be esterified during the triosephosphate oxidation to replace the A.T.P. being broken down in the reverse reaction. To explain the actual breakdown of A.T.P. observed in the presence of the arsenate one would therefore have to assume that arsenate prevents the esterification of inorganic phosphate accompanying the oxidation of triosephosphate, without affecting the reverse reaction in any way, so that the equilibrium will be upset and the A.T.P. continually decomposed but not reformed. A small amount of phosphoglycerate and cozymase would then act catalytically, being alternately reduced and then oxidized. Of course the formation of an intermediate phosphorylated cozymase both in esterification as well as in dephosphorylation would not affect the final balance of the reaction.

However, this assumption of the inhibition by arsenate of the esterification of inorganic phosphate coupled with the oxidation of triosephosphate [Needham & Pillai, 1937; Meyerhof *et al.* 1938, 1] is difficult of experimental proof because the product of the oxidation of triosephosphate itself (phosphoglycerate) would cause any adenylpyrophosphate, even if it were formed, to break down immediately in the presence of the arsenate. No other similar reactions are known, so that this action of arsenate cannot be tested elsewhere. Arsenate as far as is known only slightly inhibits the phosphatases and does not prevent the transfer of phosphate from phosphopyruvate to adenylic acid, but these are rather different reactions, not comparable with esterification of inorganic phosphate. However, it seems unwarranted to assume in the absence of clear experimental evidence that arsenate inhibits the coupled esterification of inorganic phosphate, and the explanation of the breakdown of adenylpyrophosphate in the presence of arsenate has to await further knowledge.

It is known that arsenate activates the reaction triosephosphate + pyruvate (or acetaldehyde) → phosphoglycerate + lactic acid (or alcohol) to the same rate in the absence of stoichiometric amounts of adenylic acid and inorganic phosphate as when these are present in such amount. The supposition has been advanced that this is due to the fact that the reaction goes on rapidly only when accompanied by esterification of inorganic phosphate, and that arsenate, by breaking the coupling in some manner, allows it to proceed as rapidly even without simultaneous esterification. Why the reaction should go on rapidly, in the absence of large amounts of adenylic acid or inorganic phosphate, merely because the esterification is prevented by arsenate is difficult to understand. Without any assumption of inhibition by arsenate this activation can be explained by the fact that any ester formed (A.T.P. or cozymasepyrophosphate) is decomposed rapidly in the presence of phosphoglycerate and arsenate. Since there are always traces of coenzymes as well as inorganic phosphate present, the function of the arsenate would be to regenerate the coenzyme and inorganic phosphate by rapid breakdown of the coenzymepyrophosphate formed during the oxidation of triosephosphate, so that continuous esterification may occur. The arsenate thus assures a constant supply of coenzyme and inorganic phosphate for esterification and the reaction can go on at the same rate as when large amounts of adenylic acid and phosphate are present.

The behaviour of creatinephosphate is interesting. Since it can break down

in the absence of adenylic acid it is able to transfer phosphate directly to cozymase in the presence of arsenate, Mg and the complete oxidoreduction enzymes. Phosphopyruvate however is not dephosphorylated in the absence of adenylic acid, showing that it cannot transfer phosphate directly on to cozymase but only through adenylic acid, a result in keeping with other observed facts. Further, the necessity for the presence of Mg for the dephosphorylation of creatinephosphate but not for that of A.T.P. would indicate that the function of the Mg is to facilitate the transfer of phosphate between molecules of two dissimilar substances like creatinephosphate and cozymase and that its aid is not necessary for the exchange between two closely related compounds like adenylpyrophosphate and cozymase.

Finally, it has been observed that arsenate instead of activating sometimes inhibits glycolysis [Meyerhof, 1927], and also that it inhibits esterification of glucose [see Schäffner & Krumey, 1936]. While with the higher concentrations of arsenate the inhibition might to some extent be due to injury to the enzymes, it seems likely that the arsenate, by accelerating the breakdown of cozymasepyrophosphate and adenylpyrophosphate, may prevent under certain conditions the esterification of carbohydrate by these sources and thus retard glycolysis as a whole.

Summary

1. Adenylpyrophosphate breaks down rapidly into adenylic acid and inorganic P in the presence of arsenate in muscle extract containing no adenylpyrophosphatase if cozymase, a trace of phosphoglycerate and NaF (to prevent the decomposition of the phosphoglycerate) are added.

2. This reaction is the basis of the activation of hexosediphosphate breakdown by arsenate. The activation is prominent at two stages, (1) the oxidoreduction between triosephosphate and pyruvate (or acetaldehyde), and (2) the dephosphorylation of phosphoglycerate. The first reaction is considerably activated by the presence of stoichiometric amounts of adenylic acid and inorganic P which are esterified simultaneously, and since, in the presence of arsenate, the phosphoglycerate formed by oxidation of the triosephosphate causes a rapid breakdown of any A.T.P. formed, thus constantly regenerating adenylic acid and inorganic P for continuous esterification, the reaction goes on as quickly as before even when only traces of adenylic acid and inorganic P are available. The dephosphorylation of phosphoglycerate similarly proceeds as rapidly as when an equivalent amount of adenylic acid is present.

3. The mechanism of the reaction is not known but it is possible that it involves reduction of the phosphoglycerate to triosephosphate and formation of a cozymasepyrophosphate as an intermediate phosphorylated compound.

4. Creatinephosphate can break down similarly in the presence of arsenate, cozymase, Mg and a trace of phosphoglycerate and NaF, apparently without adenylic acid as intermediary phosphate transporter.

5. This activated breakdown in the presence of arsenate of adenylpyrophosphate, which is the most important phosphorylating intermediary, might by preventing esterification of carbohydrate account for the inhibition of glycolysis by arsenate sometimes observed.

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REFERENCES

Adler & Günther (1938). *Hoppe-Seyl. Z.* **253**, 143.
Harden (1932). Alcoholic Fermentation. Longmans, Green & Co., London.
—— & Young (1906). *Proc. chem. Soc.* **22**, 283.
—— —— (1911). *Proc. roy. Soc.* B, **83**, 451.
Kiessling (1936). *Ber. dtsch. chem. Ges.* **69**, 2331.
Macfarlane (1930). *Biochem. J.* **24**, 1051.
Meyerhof (1918). *Hoppe-Seyl. Z.* **102**, 185.
—— (1921). *Pflüg. Arch. ges. Physiol.* **188**, 114.
—— (1927). *Biochem. Z.* **183**, 176.
—— (1934). *Biochem. Z.* **273**, 80.
—— (1937). *Ergebn. Physiol.* **39**, 10.
—— & Kiessling (1935). *Biochem. Z.* **280**, 99.
—— —— (1936). *Naturwissenschaften*, **24**, 361.
—— —— & Schulz (1937, 1). *Biochem. Z.* **292**, 25.
—— Ohlmeyer & Möhle (1938, 1). *Biochem. Z.* **297**, 90.
—— —— —— (1938, 2). *Biochem. Z.* **297**, 113.
—— Schulz & Schuster (1937, 2). *Biochem. Z.* **293**, 309.
Needham & Pillai (1937). *Biochem. J.* **31**, 1837.
Ohlmeyer & Ochoa (1937). *Biochem. Z.* **293**, 338.
Parnas & Lutwak-Mann (1935). *Biochem. Z.* **278**, 11.
Pett & Wynne (1934). *Biochem. J.* **28**, 365.
Pillai (1938). *Biochem. J.* **32**, 1087.
Raymond (1928). *J. biol. Chem.* **79**, 637.
Runnström & Hemberg (1937). *Naturwissenschaften*, **25**, 74.
Schäffner & Krumey (1936). *Hoppe-Seyl. Z.* **243**, 149.
Warburg, Christian & Griese (1935). *Biochem. Z.* **282**, 157.

CCLVI. THE OXIDATION PRODUCTS OF THE UNSATURATED ACIDS OF LINSEED OIL

By LESLIE CHARLES ALFRED NUNN and IDA SMEDLEY-MACLEAN
Biochemical Department, Lister Institute

(*Received 30 September 1938*)

Green & Hilditch [1935] investigated the effects of various oxidizing agents on the unsaturated acids of linseed oil and found that the best yields of hydroxy-acids were obtained by the action of alkaline permanganate, though even with this the yields were low; from linoleic acid, 40 % tetrahydroxystearic acid was obtained whilst the yield of hexahydroxy-acids from linolenic acid was only 15 to 18 % of that theoretically possible. These authors also investigated the oxidation of pure α-linoleic acid and isolated from it 65 % of the theoretical yield of tetrahydroxy-acid when they worked under the conditions described by Hazura [1888] and 38 % when the conditions given by Lapworth & Mottram [1925] were used. β-Linoleic acid oxidized under Hazura's conditions furnished only 5 % of the tetrahydroxy-compound. They concluded that the α-form of linoleic acid is the one normally present in natural oils and that the β-compound is a complex mixture of transposition products, more readily degraded by oxidation than is the original α-acid.

In 1935 we published a preliminary communication on the oxidation of the acids of linseed oil by means of alkaline permanganate: the proportions of the various hydroxy-acids formed were determined and the presence of lactonic acids established amongst the degradation products.

The methods available for the analysis of linseed oil or of any oil containing more than two unsaturated acids are still far from satisfactory. The solid bromination products obtained on bromination of linoleic and linolenic acids represent only a small proportion of the total bromides, and bromine determinations have therefore to be carried out on the various fractions of the liquid bromides. Only if the amount of one of the three unsaturated acids present is known can the proportions of the other two be calculated from the I.V. Kaufmann's "thiocyanogen number" [Kaufmann & Keller, 1929], which has been used to determine the amounts of linoleic and linolenic acids, depends on arbitrary assumptions and in the hands of different observers gives very variable results[1] [cf. Gay, 1932]. The conversion of pure oleic acid into dihydroxystearic acid is, however, almost theoretical. Lapworth & Mottram [1925] found that, working with 1 % $KMnO_4$ in very dilute solutions, a 96 % yield of the dihydroxy-acid was obtained. They found, however, that when as much as 12 to 13 % linoleic acid was present with the oleic acid, the total precipitate containing both the di- and tetra-hydroxy-acids corresponded only with 96 % of the amount of hydroxy-acid theoretically obtainable. They add: "Judging from the results of experiments which the authors have made on the production of hydroxyketostearic acid, the yield of dihydroxystearic acid may fall off very rapidly if the proportion of acids more highly unsaturated than oleic acid exceeds a certain maximum." It is not, however, stated what conditions were used in these experiments on the forma-

[1] Griffiths & Hilditch [1934] found the method quite satisfactory if sufficient care were taken to exclude all traces of moisture.

tion of the hydroxyketo-acid, but they almost certainly must have differed from those in which only the hydroxy-acids were produced.

In our experience using Rollett's [1909] conditions of oxidation, the conversion of oleic acid into dihydroxystearic acid is remarkably constant even in the presence of considerable quantities of the higher unsaturated acids.

15 g. acids obtained by the saponification of linseed oil were dissolved in 18 ml. 33 % KOH solution and 3 l. water added. The solution was cooled to 0° and 2 l. 1 % $KMnO_4$ solution previously cooled to 0° added during a period of 30 min., the mixture being constantly stirred and cooled with ice so that the temperature never rose above 5°; SO_2 was then immediately passed in until the MnO_2 was dissolved, and the solution acidified with dilute H_2SO_4. The precipitate, which at once separated, was filtered off, dried and extracted for 24 hr. with boiling light petroleum (B.P. 60–80°). The residue was then thoroughly extracted with boiling water to remove tetra- and hexa-hydroxystearic acids, dried and weighed. In three similar experiments it corresponded to 96 % of the dihydroxy-acid theoretically obtainable from the oleic acid present.

Two examples of crude oleic acid analysed in this way gave the following results.

(*a*) A commercial specimen, purified once by the Pb salt-alcohol method, I.V. 87·8. Two determinations were made, 5 g. acid being used for each. After allowing for the 0·95 g. saturated acid, the I.V. corresponded to 65 % oleic and 16·1 % linoleic acid. The percentage of oleic acid calculated from a 96 % yield of the dihydroxy-compound was 64·5.

Weight in g. of	(*a*)	(*b*)	Mean
Crude hydroxy-acid ppt.	5·14	5·24	5·19
Petrol-soluble saturated acids	0·88	1·01	0·95
Dihydroxy-acid	3·46	3·48	3·47
Tetrahydroxy-acid	0·34	0·32	0·33
Water-soluble residue	0·51	0·49	0·50

(*b*) A fraction of the unsaturated acids from butter which had been separated by distillation of the methyl esters and the unsaturated acids purified by the Pb salt-alcohol and Li salt-acetone processes gave 75·6 % oleic acid calculated from the I.V. and 75·4 % calculated from the dihydroxy-acid. Calculated from the I.V. 9·0 % linoleic acid was present.

The composition of the Calcutta linseed oil, I.V. 180, used for the oxidation experiments was investigated by the above method. After determining the proportions of saturated and oleic acids present, the amounts of linoleic and linolenic acids were calculated from the I.V. of the original mixture of acids.

	%
Saturated acids	10·90
Oleic acid	12·96
Linoleic acid	26·00
Linolenic acid	43·60
Unsaponifiable matter	1·20

Two determinations of the saturated acids, using the Pb salt-alcohol method of separation, were also made and gave 9·2 and 8·9 % saturated acids. When oxidizing larger quantities of material the best results were obtained if a preliminary separation of part of the saturated acid was first effected by applying the Pb salt-alcohol process. The unsaturated fraction was then worked up. Oxidation of 225 g. of unsaturated acids in 30 g. portions yielded hexahydroxy-acids corresponding to 39·9–51·6 % of the linolenic acid originally present and tetrahydroxy-acids corresponding to 27·6–32·3 % of the linoleic acid.

Table I. *Showing yields of oxidation products obtained from the oxidation of 225 g. linseed oil unsaturated acid fraction*

	(1) g.	(2) g.	M.P.
(1) Saturated acids	12·5	9·5	—
(2) Dihydroxystearic acid	36·0	38·2	132°
(3) Tetrahydroxystearic acid, soluble in boiling, insoluble in cold water	19·0	22·2	156–165°
(4) Hexahydroxystearic acids:			
(1) Linusic acid (obtained from aqueous extract after separation of the tetrahydroxy-acid)	36·0	49·0	180–195°
(2) *iso*Linusic acid (obtained as oily ppt. on further concentrating aqueous filtrate from ppt. of hydroxy-acids. Purified by ether extraction)	18·8	20·8	173–175°
(5) Lactonic acid. $C_{12}H_{18}O_4$. Dark oil extracted by ether from *iso*linusic fraction	—	16·7	Oil
(6) Viscid yellow oil obtained by further concentrating aqueous filtrate. Contains lactonic acid $C_{12}H_{20}O_5$, small quantities of azelaic acid and other acids	—	75·2	Oil
(7) Soluble substances readily reducing $KMnO_4$, remaining in aqueous filtrate	—	—	—
(8) Volatile acids. Formic and *n*-hexoic present and probably propionic	—	—	—

It was estimated that fractions (1) to (6) contained approximately 80% of the carbon of the acids oxidized.

The nature of the oil extracted by ether from the isolinusic *acid fraction*

This oil was not examined in our preliminary investigation.

After removal of the ether, a yellow limpid oil remained which gradually changed on standing in the desiccator to a dark brown viscous substance, sparingly soluble in ether, some form of polymerization possibly taking place. The oil was extracted with benzene and the portion soluble both in ether and in benzene examined.

	Analysis of fractions			Equivalent	
	% C	% H	I.V.	Neutralization	Saponification
(1) Benzene- and ether-insoluble	65·60	8·60	18·8	—	176·9
(2) Benzene-insoluble, ether-soluble	62·20	7·59	19·6	262·0	168·9
(3) Benzene- and ether-soluble	63·47	7·92	27·6	236·6	159·4
Theory for $C_{12}H_{18}O_4$	63·70	8·10	112·0	226·0	122·0

The clear yellow solution on standing deposited a brown viscous oil insoluble in benzene, so that polymerization possibly again took place in the benzene solution.

The difference between the equivalents obtained by direct titration and by saponification suggested the presence of a large proportion of lactonic acids. The analysis of the benzene-soluble oil agreed with the formula $C_{12}H_{18}O_4$; the difference between the neutralization and saponification values indicated the presence of the lactone of a hydroxy-dibasic acid. The actual determination of the end-point of the saponification value was difficult owing to the dark brown colour of the solution after boiling with alcoholic KOH. The discrepancy was, however, too big to be entirely explained in this way, and either the lactone ring was incompletely opened after 1 hr. boiling with alcoholic KOH or else some monobasic acid was present as impurity. The low I.V. obtained is typical of the

behaviour of the $\alpha\beta$-unsaturated acids. These data indicate that the constitution of the ether- and benzene-soluble oil is as follows:

$$\begin{array}{l} OC.HC{:}CH.CH.(CH_2)_7.COOH. \\ \quad\quad\quad\

was precipitated and it did not behave as a homogeneous substance. On acidifying the solution, an oily acid separated which gradually deposited a small amount of crystals of azelaic acid. Possibly an unstable double salt of Zn azelaate with the Zn salt of a higher acid is formed. From the evidence now available it seems probable that this fraction contained the lactonic acid of the β-hydroxy-acid corresponding with the unsaturated lactonic acid described above. We cannot confirm the isolation of the Zn salt of the dihydroxydibasic acid: in the light of further experience, it seems probable that the free $\beta\gamma$-dihydroxydibasic acid would be at once converted into the corresponding lactonic acid.

The oxidation of linoleic acid

In the first experiment, 90 g. linoleic acid were obtained from the solid tetrabromide isolated from the bromination of the unsaturated acids of maize oil [Nicolet & Cox, 1922]. The I.V. was 150 and it contained about 9 % saturated acids. The procedure already described for the oxidation of the linseed oil acids was followed for the oxidation of the linoleic acid. After the precipitate containing the tetrahydroxystearic acid and the saturated acids had been separated, the solution was further concentrated until the approximate concentration at which the *iso*linusic fraction separated from the oxidation product of the linseed oil acids had been reached. An oil then separated, completely soluble in ether and amounting to 11·4 % of the original weight of linoleic acid taken. Further concentration resulted in the precipitation of 12·2 g. of an oil from which a small quantity of azelaic acid (1·2 %) was crystallized.

90 % of the oil corresponding in its water-solubility with the "*iso*linusic fraction" was extracted with benzene and possessed the following properties:

	% C	% H	I.V.	Equivalents: Neutralization	Equivalents: Saponification
Benzene-soluble oil	65·88	9·53	2·05	272	184
Corresponding fraction from linseed oil acids	63·47	7·92	27·6	236·6	159·4

The benzene-soluble oil contained, therefore, some compound with a higher percentage of carbon than that of the C_{12} lactonic acid.

Determination of hydroxyl group

The lactone was esterified and the hydroxyl value of the ethyl ester estimated by the Zerewitinoff method. 0·2841 g. ethyl ester gave 12·28 ml. CH_4 at N.T.P. This would correspond with the presence in the oil of 55 % of the dibasic acid $C_{12}H_{20}O_5$ formed by opening the lactone ring.

Reduction

(*a*) *By sodium amalgam.* After treating with sodium amalgam in faintly acid solution for 12 hr., micro-determinations of the C and H contents of the oil showed that the substance was unaltered. No readily reducible ethylene linkage was present in the molecule.

(*b*) *By platinum oxide and hydrogen.* 0·45 g. absorbed 25 ml. hydrogen (N.T.P.). The equiv. wt. of the lactonic acid is 226 and 226 g. oil had absorbed 12·55 l., i.e. 0·56 mol. hydrogen. This determination together with the low I.V. (2·03) suggested the presence of 55 % of a substance containing an $\alpha\beta$-ethylene linkage; the close agreement of this figure with that obtained in the determination of the

hydroxyl group suggested that 55 % of the $\alpha\beta$-unsaturated lactonic acid was present together with 45 % of some saturated non-hydroxylated substance containing a higher percentage of carbon. The product gave no precipitate with dinitrophenylhydrazine so that the presence of a keto-group could be excluded. Since our starting material was linoleic acid and the product was saturated, dioxidostearic acid presented itself as a possible constituent, as it is saturated and contains no hydroxyl group.

The carbon and hydrogen percentages and the neutralization and saponification equivalents of a mixture of 45 % dioxidostearic acid with 55 % of the lactone of hydroxy(3)-decenic-(1:2)-dicarboxylic acid (1 : 10) would be:

			Equivalent	
	% C	% H	By neutralization	By saponification
Theory	66·07	9·04	264·7	202·5
Benzene-soluble oil	65·88	9·53	—	—
	65·55	9·57	272	184

These results appeared sufficiently near to justify the conclusion that the oil consisted of a mixture of these two substances. We endeavoured to confirm this by reducing the mixture with HI and isolating the completely reduced acids.

Reduction with HI in boiling acetic anhydride [Crowder & Anderson, 1932]. 0·7 g. oil was boiled for 3 hr. with HI (sp. gr. 1·7) and red phosphorus in acetic anhydride solution. From the reaction product treated according to the directions given, stearic acid, M.P. 68°, was isolated by extraction with light petroleum and its identity confirmed by mixed M.P. From the petroleum-insoluble fraction a very small quantity of glistening white crystals soluble in hot water was obtained: these melted at 122–124°. *n*-Decanedicarboxylic acid was synthesized from ω-bromoundecanic acid, for a specimen of which we are indebted to Dr J. C. Smith; the dicarboxylic acid melted at 125–126°, and admixture with the specimen obtained by reduction of the lactonic acid gave no depression. The crystals gave no evidence of lactonic properties. The presence of a C_{18} compound and of a derivative of the C_{12} dicarboxylic acid was thus confirmed. 0·7 g. dioxidostearic acid similarly reduced yielded 0·22 g. stearic acid.

The lactonic acid $C_{12}H_{18}O_4$ obtained as an oxidation product of the mixed linseed oil acids has, therefore, been identified amongst the oxidation products of pure linoleic acid. The presence of dioxidostearic acid appears not to have been previously noticed among the products of oxidation of linoleic acid by alkaline permanganate, although it is formed when oxidation is carried out by perbenzoic acid [Green & Hilditch, 1935].

Proportion of linoleic acid recovered as tetrahydroxy-acid under Rollett's conditions of oxidation

In the first experiment carried out 90 g. linoleic acid (I.V. 150) (from which the saturated acid had not been completely removed) were oxidized. From the I.V. the proportion of pure linoleic acid was 83·1 %; the weight of pure acid oxidized was therefore 74·8 g.; 50 g. tetrahydroxy-acid were isolated—a yield of 53·8 %. In a second experiment, 30 g. linoleic acid, I.V. 166·7, were oxidized and 18·1 g. tetrahydroxy-acid recovered, a yield of 52·8 %. The percentages of linoleic acid degraded were therefore 46·2 and 47·2. Green & Hilditch [1935] found a 65 % yield of the tetrahydroxy-acid using Hazura's [1888] conditions, and 38 %

when the conditions described by Lapworth & Mottram [1925] were employed. As we only isolated 27·6–32·3 % tetrahydroxy-acid from the linseed oil mixed acids, we confirm Green and Hilditch's results that when the mixed unsaturated acids are oxidized, a far larger proportion of the tetrahydroxy-acid is broken down than when pure linoleic acid is oxidized. In view of the finding of these authors, that the oxidation products of β-linoleic acid are almost completely degraded to lower carbon compounds, possibly the effect of the presence of linolenic acid is to increase the isomerization of linoleic acid from the α- to the β-form.

Oxidation of linolenic acid

Two experiments were carried out using linolenic acid prepared from the solid hexabromide obtained in the bromination of the linseed oil acids. The I.V. was 258 corresponding to 94·1 % of pure linolenic acid. 15 g. quantities were used for the oxidations: the yields of linusic acids were respectively 10·7 and 8·3 % and of *iso*linusic 14·7 and 14·0, making a total percentage yield of hexahydroxy-acids of 25·4 and 22·3 %. Green and Hilditch give 18 % as their yield. When the pure linolenic acid was oxidized about 75% was degraded to compounds containing smaller numbers of carbon atoms, but when the mixed linseed oils were oxidized the proportion of linolenic acid broken down to lower carbon compounds was only 55–64 %. The linoleic acid in the mixture appears, therefore, to exercise a protective action on the linolenic acid.

Conclusions

1. The products of oxidation of the mixed acids of linseed oil by means of alkaline permanganate under Rollett's conditions have been investigated.
2. The proportion of the various oxidation products has been determined.
3. The 96 % yield of dihydroxy-acid obtained from oleic acid under these conditions was very little affected by the proportions of linoleic and linolenic acid present.
4. The estimation of oleic acid in a mixture of unsaturated acids is most conveniently made as dihydroxystearic acid.
5. The yields of tetrahydroxy-acids from the linoleic acid present in the mixed linseed oil acids were only 28–32 %: yields of 52–54 % were obtained when pure linoleic acid was similarly oxidized.
6. The yields of hexahydroxy-acids calculated on the linolenic acid present in the mixed acids were 40–52 %. When pure linolenic acid was similarly oxidized, only 22–25 % hexahydroxy-acid was separated. Linoleic acid is therefore more readily degraded in the presence of linolenic acid and exercises a protective action on the oxidation of the linolenic acid.
7. The greater part of the degradation products consists of γ-lactonic acids containing 12 carbon atoms. The presence of the acid $C_{12}H_{18}O_4$ has been established and its constitution determined as the γ-lactone of hydroxy-(3)-decenic (1:2)-dicarboxylic acid (1:10). The same acid has been shown to occur amongst the degradation products of pure linoleic acid.
8. Evidence of the existence of the γ-lactone of dihydroxy-(3:4)-decane-dicarboxylic acid has been obtained.
9. The amount of azelaic acid formed on oxidation under the given conditions is very small (1·2 %).
10. Fission takes place more readily between the 12:13 carbon atoms carrying the hydroxyl groups than between those in the 9:10 positions.

The micro-determinations were carried out for us in Dr Weiler's laboratory. One of us (L. C. A. N.) desires to acknowledge his gratitude to the Department of Scientific and Industrial Research for a grant enabling him to carry out this work.

REFERENCES

Burr & Burr (1929). *J. Biol. Chem.* **82**, 345.
Crowder & Anderson (1932). *J. Biol. Chem.* **97**, 399.
Gay (1932). *J. Soc. chem. Ind.* 126 T.
Green & Hilditch (1935). *J. Biochem.* **29**, 1552.
Griffiths & Hilditch (1934). *J. Soc. Chem. Ind.* **53**, 75 T.
Hazura (1888). *Monatsh. Chem.* **9**, 180.
Kaufmann & Keller (1929). *Z. angew. Chem.* **42**, 20, 73.
Lapworth & Mottram (1925). *Trans. Chem. Soc.* p. 1628.
Nicolet & Cox (1922). *J. Amer. chem. Soc.* **44**, 144.
Nunn & Smedley-Maclean (1935). *J. Biochem.* **29**, 2742.
Rollett (1909). *Hoppe-Seyl. Z.* **62**, 410.

CCLVII. EXPERIMENTS ON THE CHEMOTHERAPY OF CANCER

II. THE EFFECT OF ALDEHYDES AND GLUCOSIDES

By ERIC BOYLAND and ELINOR HUNTSMAN MAWSON

From the Research Institute of the Royal Cancer Hospital

(*Received 28 September 1938*)

In a series of recent papers Strong [1932; 1934; 1935; 1936, 1, 2] recorded that the feeding of natural oil of gaultheria inhibited the growth of spontaneous tumours in mice. Synthetic methyl salicylate had no such effect. Strong [1938, 1] showed that the effective substance was in the low-boiling fraction of the oil, and later that it was probably heptaldehyde [Strong, 1938, 2]. For this reason a number of aldehydes and ketones have been tested for their inhibitory action on the growth of grafted tumours in mice. The more promising compounds, citral and phloroglucinol aldehyde, have been tested on spontaneous tumours in mice.

The ease with which glucose penetrates into tumour cells made it appear possible that glucosides might be preferentially absorbed by tumour tissue and for this reason some available glucosides have been administered to mice with grafted tumours. None of them appeared to be effective in inhibiting growth and they were not tested on mice with spontaneous tumours.

Experiments with the Crocker Sarcoma 180

Substances were tested on groups of 5 mice with Crocker Sarcoma 180 as previously described [Boyland, 1938]; all compounds were given by oral injection 5 or 6 times weekly. The dosing was commenced immediately after grafting of the tumours, which were grafted into pure-strain mice of either the Strong A or Little Dilute Brown (dba) strains. The tumours were measured thrice weekly and their growth compared with that of control tumours, which were growing in the same strain of mice grafted at the same time from the same tumour. Figs. 1, 4 and 7 show typical growth curves for the Crocker Sarcoma 180 in Strong A, Dilute Brown, and in stock mice. In stock mice the rate of growth in different mice is so variable that it is almost impossible to show the effect of inhibitory substances unless large numbers of mice are used. It is for this reason that mice from pure lines have been used for all the experiments with grafted tumours which are described in this paper. The compounds tested were administered in doses which would not injure the mice. The positive effects appeared to be of two kinds indicated in Table I.

Retardation in the rate of growth of the tumour as compared with that of the controls is indicated by *R*.

Increase in the length of life of the animals without effect on the rate of growth is indicated by *L*.

When no indication is made in the table the tumours in the treated animals were not significantly different from the tumours in control mice.

Table I. *The effect of orally administered substances on the Crocker Sarcoma 180 in Dilute Brown or Strong A mice*

R = retardation of tumour growth. *L* = increase in length of life.

Substance	Dose administered daily mg.	Effect
Anisaldehyde	5	—
o-Chlorobenzaldehyde	5	—
m-Chlorobenzaldehyde	5	—
p-Chlorobenzaldehyde	5	—
Cinnamic aldehyde	5	—
Citral	10	*R*
3:4-Dimethoxybenzaldehyde	5	*R*
p-Dimethylaminobenzaldehyde	5	—
3:4-Diethoxybenzaldehyde	5	—
Gentisic aldehyde	4	—
Heptaldehyde	5	—
,,	50	—
o-Hydroxybenzaldehyde (salicylaldehyde)	4	—
m-Hydroxybenzaldehyde	4	—
p-Hydroxybenzaldehyde	4	—
2-Hydroxy-6-*tert*-butylbenzaldehyde	5	—
o-Methoxybenzaldehyde	5	*L*
2-Naphthol-1-aldehyde	10	—
6-Nitro-3-hydroxybenzaldehyde	5	—
Octaldehyde	5	—
Phloroglucinaldehyde	5	*R*
Resorcaldehyde	4	—
3:4:5-Trimethoxybenzaldehyde	5	—
Valeraldehyde	10	—
Oils:		
Oil of Wintergreen (from *Betula lenta*)	10	—
Crude oil of Betula	20	—
Refined oil of Betula	20	—
Betulol	20	—
Glucosides:		
Aesculin	10	—
Amygdalin	10	—
Arbutin	20	*L*
Helicin	10	—
Salicin	10	*L*
Ketones:		
Acetophenone	10	—
Dimedone	5	—
Methylheptanone	10	*R*
Methylheptenone	10	—

Retardation of growth was obtained with citral, phloroglucinaldehyde and 3:4-dimethoxybenzaldehyde. The effect with phloroglucinaldehyde is shown by comparison of Figs. 1 and 3. Fig. 2 shows the growth of grafted tumours in mice treated with heptaldehyde. Other experiments with heptaldehyde also showed no inhibition of the growth of grafted tumours. The results with phloroglucinaldehyde and citral were of sufficient promise to make it seem worth while to test related compounds.

The prolongation of life observed in mice treated with *o*-methoxybenzaldehyde and salicin is illustrated in Figs. 5 and 6 as compared with the controls in Fig. 4. The effect may be due to antiseptic action of these compounds reducing the toxic effects of any contamination which may be present in the grafted tumour.

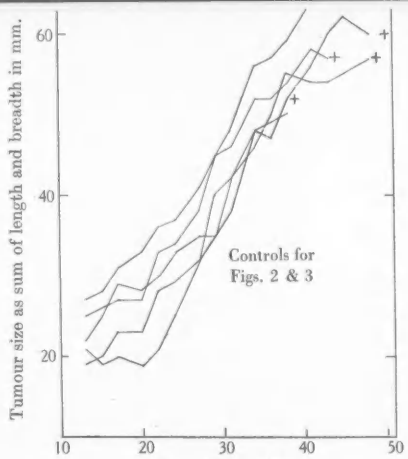

Fig. 1.

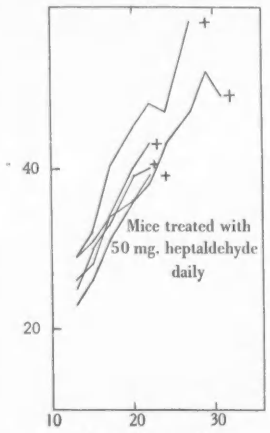

Fig. 2.

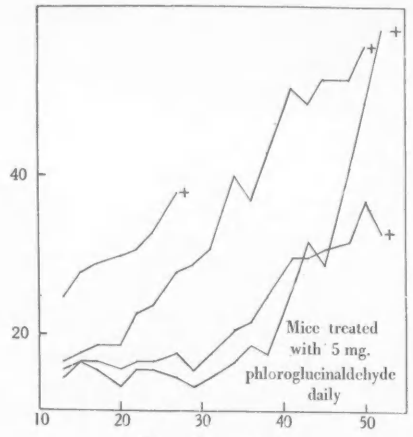

Fig. 3.

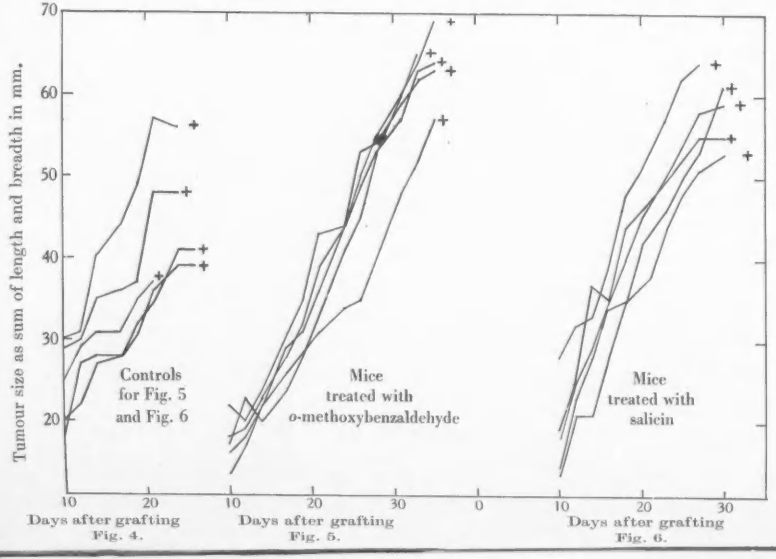

Fig. 4. Fig. 5. Fig. 6.

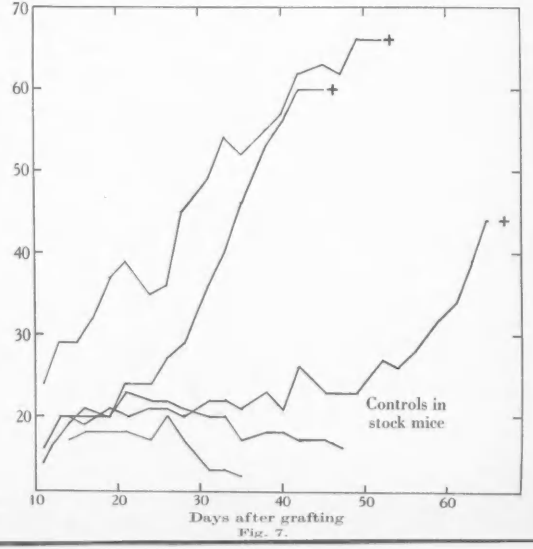

Fig. 7.

Days after grafting
Fig. 4.

Days after grafting
Fig. 5.

Days after grafting
Fig. 6.

Days after grafting
Fig. 7.

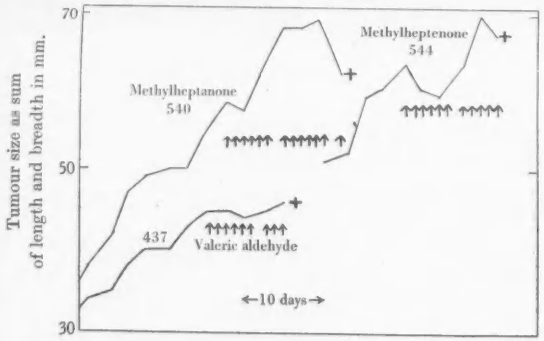

Fig. 9.

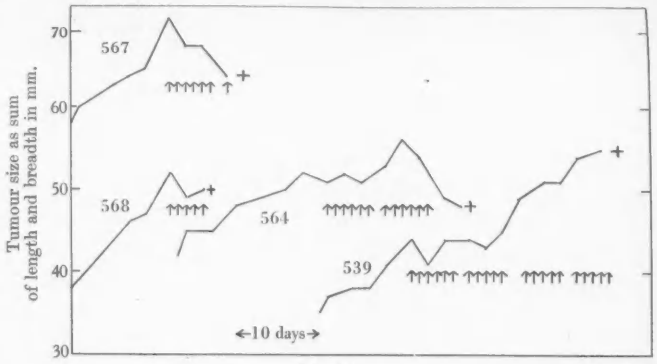

Fig. 10.

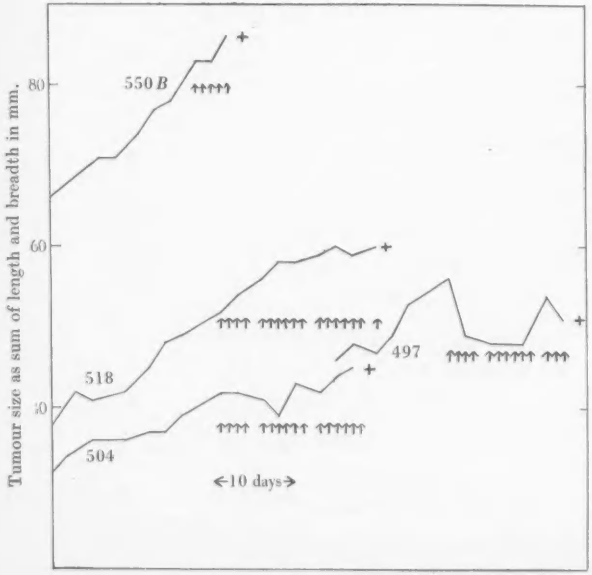

Fig. 8.

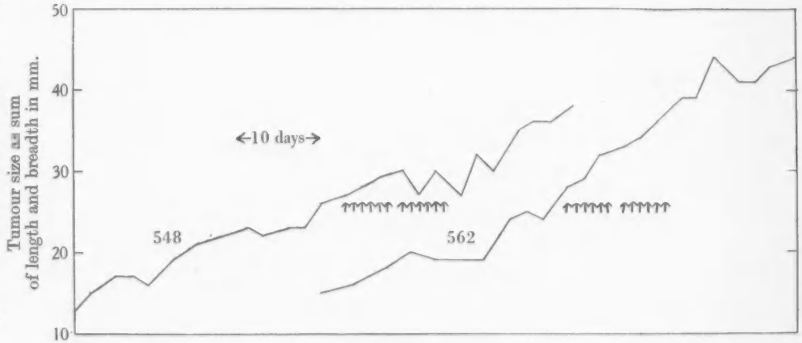

Fig. 11.

Figs. 1–3. Growth of Crocker Sarcoma 180 in Strong A mice.

Figs. 4–7. Growth of Crocker Sarcoma 180 in mice. Figs. 4–6 in Dilute Brown mice. Fig. 7 in Stock mice.

Fig. 8. Growth of spontaneous mammary tumours in mice treated with heptaldehyde (50 mg. per day).

Fig. 9. Growth of spontaneous mammary tumours in treated mice.

Fig. 10. Growth of spontaneous mammary tumours in mice treated with citral.

Fig. 11. Growth of spontaneous mammary tumours in mice treated with phloroglucinaldehyde.

Experiments with spontaneous mammary tumours

Some experiments were carried out on spontaneous mouse tumours under the same conditions as previously described [Boyland, 1938]. Unless otherwise stated the dose was that given in Table I. Fig. 8 shows the experiment with heptaldehyde. In the case of one mouse (497) treatment caused partial retrogression and in mice 504 and 518 retardation of growth. Methyl heptanone and methyl heptenone were each tried on one tumour (Fig. 9), and the latter produced a temporary retrogression when first administered. Valeraldehyde (Fig. 9) caused inhibition of tumour growth but the mouse did not survive the treatment very long.

Experiments with citral and phloroglucinaldehyde, which were the two most effective aldehydes tested with the Crocker Sarcoma, are shown in Figs. 10 and 11. Citral produced partial retrogression in two mice and inhibition of growth of the other two tumours used. Phloroglucinaldehyde inhibited one of the two tumours on which it was tried.

An experiment was carried out to determine the effect of heptaldehyde on growth in body wt. of normal young mice. The groups of 4 mice each of the average wt., 11·3 and 13·5 g. respectively, were weighed daily. In one group each mouse was given 50 mg. heptaldehyde daily while the other group was left as controls. At the end of 20 days the average wt. of the first group was 19·5 g., while that of the second group was 20·5 g. The heptaldehyde did not affect body growth.

Discussion

Of the series of aldehydes and ketones which have been tested, citral caused some inhibition of growth of both grafted and spontaneous tumours. Strong (personal communication) found that citral had some inhibitory action on spontaneous tumours in 1931. Heptaldehyde inhibited spontaneous tumours but not grafted tumours. The relative ineffectiveness of heptaldehyde in inhibiting grafted tumours while it inhibits spontaneous tumours to some extent is of interest in that its action is the converse of that of colchicine. Colchicine produces haemorrhage and some inhibition of grafted tumours but not of spontaneous tumours [Boyland & Boyland, 1937].

It is conceivable that the action of aldehydes is due to peroxide formation. Aldehydes are known to form peroxides on exposure to air and it is possible that different samples might contain varying amounts of organic peroxide. Maisin *et al.* [1938] have described the anticarcinogenic action of formaldehyde peroxides and the effect of heptaldehyde may conceivably be due to similar products.

Summary

A number of aldehydes, ketones and glucosides have been tested for inhibitory action on tumour growth. Of these citral and heptaldehyde inhibited the growth of spontaneous tumours in mice.

We should like to thank Mr Sharp and Dr T. A. Henry of the Wellcome Research Laboratories for many of the aromatic aldehydes, Prof. Cook for samples of citral, methyl heptenone and methyl heptanone and for suggesting that these compounds might be tested, and Mrs Robinson for phloroglucinaldehyde.

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REFERENCES

Boyland (1938). *Biochem. J.* **32**, 1207.
— & Boyland (1937). *Biochem. J.* **31**, 454.
Maisin, Pourbaix & Caeymaex (1938). *C.R. Soc. Biol., Paris*, **127**, 1479.
Strong (1932). *Proc. Soc. Exp. Biol., N.Y.*, **30**, 386.
— (1934). *Amer. J. Cancer*, **20**, 387.
— (1935). *Amer. J. Cancer*, **25**, 797.
— (1936, 1). *Amer. J. med. Sci.* **192**, 546.
— (1936, 2). *Amer. J. Cancer*, **28**, 550.
— (1938, 1). *Amer. J. Cancer*, **32**, 227.
— (1938, 2). *Science*, **87**, 184.

CCLVIII. OBSERVATIONS ON SPECIFIC NUTRITIONAL FACTORS IN LACTATION

By SYDNEY JOHN FOLLEY, ELIZABETH WOODROFFE IKIN, STANISŁAW KAZIMIERZ KON and HELEN MARGARET SCOTT WATSON

From the National Institute for Research in Dairying, University of Reading

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It is well known that diet has a profound effect on lactation and that the satisfactory secretion of milk is only possible in the presence of certain known dietary factors in adequate quantities. There is also general agreement that a diet good enough to support the physiological strain of optimal lactation would also satisfy the requirements for optimal growth and reproduction.

The converse, however, does not seem to be necessarily true; in experiments on rats several authors have found that diets made up from natural foodstuffs which allowed, under stock colony conditions, of excellent growth and reproduction were very unsatisfactory with regard to the lactational performance of the animals [e.g. Smith & Anderson, 1929; Kon, 1931; Kon & Peskett, 1933; Coward, 1938]. Such findings obviously suggest the existence of separate dietary factors specifically connected with lactation, and publications to this effect are not lacking. A number of workers, who used so-called synthetic diets and included in them all dietary factors known by them to be required by the rat, have reported lactational failures, though otherwise the performance of the animals was very good. Efforts to find out the cause of the trouble have shown in several instances that this was nutritional and could be cured by supplying the rats with certain natural substances or with extracts from them which apparently contained a distinct new dietary principle capable of stimulating lactation. Thus, Mapson [1932; 1933] showed that a supposedly complete synthetic ration could be much improved as regards its value in lactation by the addition of fresh ox liver or of aqueous extracts thereof. It should be noted that liver exerted also a marked stimulating effect on the growth of young rats fed on the synthetic diet, but it is not known whether the two actions were due to the same or to different substances.

Similar observations on the favourable effect of liver in conjunction with semi-synthetic diets containing varying proportions of soya-beans were made by Wilkinson & Nelson [1931] and studies by Feaster & Nelson [1935] also showed the value of liver and led the authors to assume the existence of a new dietary lactational factor.

Nakahara and his colleagues [Nakahara & Inukai, 1933; 1934; Nakahara *et al.* 1934; 1935; 1936; 1937; 1938] have published a comprehensive series of papers in which they reported failure of lactation in rats kept on a presumably complete synthetic diet and marked improvement following the administration of fresh liver or of carefully fractionated liver extracts.

In addition to liver, other natural foods have been found to supply factors capable of stimulating lactation. Thus Tso [1927] and Outhouse [1937] found these present in lettuce, and the latter and also Meyer [1936] found them in

oats and to a lesser extent in hydrogenated cotton seed oil. It was shown that vitamin E was not involved.

Swanson & Nelson [1937], who used synthetic diets containing dried tinned pork muscle as the sole source of protein, were able to cut down considerably the number of lactational failures on such diets by the administration of fresh liver, but found another substance, the pancreatic hormone "lipocaic" of Dragstedt *et al.* [1936], to be extremely active in this respect.

These admittedly incomplete quotations from the literature show that many students of nutrition have had little success with their purified diets when lactational performance was considered, even though an effort was made to include in such diets all known essential food constituents.

When weighing up the evidence, however, the remarkable experiments of Cox & Imboden [1936, 1] should be placed on the other side of the scale. These authors fed in the course of 2 years, to three generations of rats, exclusively a conventional "synthetic" diet and obtained remarkable success in growth, reproduction and lactation, all of which were as satisfactory as on their good stock diet. A large group of female rats brought to maturity on the stock diet was put through 10 reproductive cycles on the synthetic diet and lactation in this case was also very satisfactory [Cox & Imboden, 1936, 2]. The diet consisted of casein, dextrin, lard, rice-cellulose and salts. The vitamin B complex was supplied as an aqueous, protein-free extract from yeast and the other addenda were wheat germ oil and an oily solution of carotene. Vitamin D was not supplied separately but it seems probable that the 9% of lard contained enough of the factor [Kon & Booth, 1934]. Richter *et al.* [1938] also noticed successful lactation when rats were offered a free selection of purified food constituents.

In planning our own investigations on the lactational performance of rats on purified diets we decided to use a diet similar to that of Nakahara & Inukai [1933] which has the advantage of simplicity and gave in the hands of these authors a high incidence of lactational failures.

Experimental

First series of experiments

Forty female rats (hooded Norwegian), born in the first half of April 1936, were reared on the stock colony diet until they were about 3 months old. This diet has the following composition:

Whole wheat, milled	69	Butter	5
Linseed cake	14	$CaCO_3$	0·5
Crude casein	6	NaCl	0·5
Dried brewer's yeast	5		

In addition the stock rats receive fresh whole milk *ad libitum*, carrots or watercress daily, liver twice a week, about 5 g. each time. Pregnant and lactating females receive liver 3 times a week. The experimental rats were then placed on the following diet:

Ground rice (Patna polished)	75
Casein (New Zealand, repeatedly washed at the isoelectric point)	10
Butter (Empire)	10
Salts (Steenbock's 40)	5
Dried yeast (D.C.L.)	5

Table I. *Lactational performance of rats on the experimental (casein) diet and on the same diet supplemented with liver, liver extract and additional yeast*

Control group

Rat no.	1st lactation. Experimental diet only: No. of pups Original ♂	Original ♀	Weaned ♂	Weaned ♀	Av. wt. at weaning ♂	Av. wt. at weaning ♀	Gain or loss in mother's wt. during lactation	2nd lactation. Experimental diet only: No. of pups Original ♂	Original ♀	Weaned ♂	Weaned ♀	Av. wt. at weaning ♂	Av. wt. at weaning ♀	Gain or loss in mother's wt. during lactation
5328	4	4	4	2	42·7	39·0	+ 4	4	4	0	0	—	—	—
5351	5	3	4	3	39·2	38·7	− 7	3	2	3	2	45·0	41·0	0
5364	5	3	5	3	35·0	34·3	+ 2	2	6	1	1	35·0	29·0	+19
5397	3	5	3	5	40·7	41·4	+31	3 (+5)	0	3 (+5)	0	41·2	0	−13
5412	4	4	3	3	44·0	46·0	+12	3	5	3	5	39·0	37·4	+ 9
5427	4	4	4	4	44·5	41·7	− 8	—*	—	—	—	—	—	—
5444	4	4	4	4	42·2	39·5	−13	4	4	4	4	41·0	37·5	− 8
5460	4	4	0	0	—	—	—	—†	—	—	—	—	—	—
5464	4	4	0	0	—	—	—	5	3	0	0	—	—	—
5485	4	4	4	4	34·7	36·0	+ 5	4	4	4	4	43·2	42·2	+19
Average	4·1	3·9	3·1	2·8	40·4	35·1	+ 3·3	4·1	3·5	2·9	2·0	40·8	37·4	+ 4·3
% of pups weaned			75·6	71·8						69·7	57·1			
			73·7							63·4				
% of does lactating successfully:			80							75				

Liver group

Rat no.	1st lactation. Experimental diet + liver: No. of pups Original ♂	Original ♀	Weaned ♂	Weaned ♀	Av. wt. at weaning ♂	Av. wt. at weaning ♀	Gain or loss in mother's wt. during lactation	2nd lactation. Experimental diet only: No. of pups Original ♂	Original ♀	Weaned ♂	Weaned ♀	Av. wt. at weaning ♂	Av. wt. at weaning ♀	Gain or loss in mother's wt. during lactation
5325	4	4	3	3	48·3	47·7	+14	3	5	3	5	44·3	42·2	+ 9
5388	4	4	4	4	44·2	41·7	+15	4	4	4	4	41·0	37·0	+19
5413	5	3	5	3	38·8	40·7	− 4	4	4	4	4	43·5	39·5	+ 2
5428	4	4	2	4	48·5	44·5	+20	3 (+1)	4	3 (+1)	4	40·0	40·0	− 3
5434	4	4	4	4	46·5	44·5	+14	5	2	4	2	50·2	46·0	−40
5437	4	4	0	0	—	—	—	3	4 (+1)	0	0	—	—	—
5343	—‡	—	—	—	—	—	—	—	—	—	—	—	—	—
5452	4	4	3	3	45·7	40·7	+19	—‡	—	—	—	—	—	—
5471	5	3	0	0	—	—	—	3	5	0	0	—	—	—
5484	4	4	3	4	44·7	40·7	+12	1 (+1)	1 (+5)	1 (+1)	1 (+4)	37·5	38·2	− 8
Average	4·2	3·7	2·6	2·7	45·3	42·9	+12·9	3·5	4·4	2·6	3·0	42·8	40·5	− 3·5
% of pups weaned			63·2	73·5						75·0	68·6			
			68·4							71·8				
% of does lactating successfully:			78							75				

Liver extract group

	1st lactation. Experimental diet + liver extract							2nd lactation. Experimental diet only						
5323	0	2	0	0	—	—	—	3	5	0	0	—	—	—
5352	4	4	1	2	32·0	34·5	+ 4	2	5	0	0	—	—	—
5356	2	6	2	5	37·5	37·2	−11	5	3	5	3	47·2	43·3	+17
5365	—†	—	—	—	—	—	—	2 (+1)	5	0	0	—	—	—
5395	4	4	0	0	—	—	—	3	5	0	0	—	—	—
5436	4	4	4	4	38·0	36·2	− 5	4	4	4	1	45·2	42·0	+13
5445	4	4	4	3	38·5	35·7	− 9	2	6	2	6	42·0	39·0	+ 5
5459	4	4	4	3	39·0	39·3	− 3	4	4	4	0	47·5	—	+13
5473	4	4	0	0	—	—	—	3	5	0	0	—	—	—
5478	4	4	4	4	39·0	34·2	−17	4	4	3	4	44·3	43·25	+ 6
Average	3·3	4·0	2·1	2·3	37·3	36·2	− 6·8	3·3	4·6	1·8	1·4	45·3	41·9	+10·8
% of pups weaned			63·3	58·3						54·5	30·4			
			60·8							42·5				
% of does lactating successfully:			67							50				

Yeast group

	1st lactation. Experimental diet + yeast							2nd lactation. Experimental diet only						
5321	4	4	2	3	42·0	39·7	+12	4	4	0	0	—	—	—
5350	4	4	0	2	—	36·0	+46	4	4	4	4	45·5	44·0	0
5394	5	3	5	3	45·2	43·0	− 2	3	4 (+1)	3	4 (+1)	45·0	41·2	−10
5396	4	4	3	4	42·0	40·5	− 7	4	4	4	3	40·7	41·3	− 2
5404	3	5	3	5	37·3	38·4	+16	3	5	3	4	37·0	35·5	−41
5435	7	1	6	1	42·7	43·0	−10	6	2	0	0	—	—	—
5443	4	4	0	0	—	—	—	—†	—	—	—	—	—	—
5451	2	3	0	0	—	—	—	—†	—	—	—	—	—	—
5472	4	4	3	2	40·7	40·0	+ 6	4	4	0	0	—	—	—
5474	5	3	0	0	—	—	—	2	2	0	0	—	—	—
Average	4·2	3·5	2·2	2·0	41·7	40·1	+ 8·7	3·8	3·8	1·8	2·0	42·1	40·5	−13·3
% of pups weaned			52·4	57·1						46·7	53·3			
			54·8							50·0				
% of does lactating successfully:			70							50				

After birth the no. of pups in the litter was reduced to 8, the 4 heaviest of each sex being kept. If the no. of live pups was under 8, substitutes were given when possible to make up this number.

* Resorption. † Doe not pregnant—too late to be remated. ‡ Died during pregnancy.

N.B. In the "no. of pups" column + indicates substituted offspring.

This diet differs from that of the Japanese workers in that casein replaces an equivalent quantity of "fish protein". Mating was started after the does had been 1 week on the diet. Females in oestrus were paired with stock colony bucks, positive matings were detected on the following morning by the vaginal smear technique and inseminated does were placed at once under the various experimental treatments. The matings were completed within a week. The distribution of the rats was as follows: 10 does were kept on the experimental diet alone and formed the control group; 10 received, in addition to the diet, 1 g. daily of fresh beef liver (liver group); 10 were similarly given daily 1 ml. of liver extract equivalent to 5 g. of fresh liver (liver extract group). The extract was prepared by the method described by Nakahara *et al.* [1935] save that the preparation was stopped short at the fractionation by phosphotungstic acid. Finally, a last (yeast) group of 10 rats was given additional yeast to ensure that the vitamin B complex was not a limiting factor in the other diets. The yeast was supplied by mixing 90 parts of the basal diet with 10 parts of a potent brewer's yeast. On the 20th day of pregnancy the rats were placed in individual cages. If more numerous, the litters were reduced to 8 on the day of birth. In the case of smaller litters this number was, if possible, made up by using young from other mothers. The young rats were weaned at 21 days. All mothers were then transferred to the basal diet only and were kept on it to the end of the experiment. They were remated about 2 months after weaning and were allowed to produce and nurse a second litter. The results are given in Table I.

It is evident from the table that we were unable to repeat the results of the Japanese workers. Not only was lactation quite satisfactory on the basal diet alone, inasmuch as 8 out of 10 does nursed their young and 74% of the available young were weaned, but none of the additions tried (fresh liver, liver extract and yeast) improved their performance. If anything, the performance of the rats in the supplemented groups was below that of the controls.

With the second matings the results were very similar. This time the rats which had previously received fresh liver fared best, but there was little to choose between them and the group which was on the control diet throughout. The "liver extract" and "yeast" groups again lagged behind.

Second series of experiments

As casein was used in our diet instead of the "fish protein" fed by Nakahara & Inukai [1933], it was thought advisable to attempt to repeat the findings of these workers by using a diet as similar to theirs as possible. For this purpose white fish ("rock salmon") was obtained from a fishmonger. The muscle was freed from visible fat and skin and, after mincing, 10 lb. were boiled for 10 min. with 9 gal. of water containing a few drops of glacial acetic acid. The solids were allowed to settle, the supernatant liquid was poured off and the process was repeated with three further lots of water. On the last occasion the solids were strained off through muslin, dried in a hot air oven and milled. This protein was used in the experimental diet, which now had the same gross composition as that of Nakahara & Inukai [1933].

Twenty-six young does born in the stock colony towards the end of December 1936 were used for this experiment. They were placed at weaning on the experimental diet described on p. 1989 and 3 weeks later were given the fish protein diet. They were thereafter kept on this diet to the end of the experiment.

As our first aim was to find out whether we could obtain a satisfactory percentage of lactational failures on a synthetic diet, no curative or protective treatments were attempted, but all the available animals were put through

Table II. *Lactational performance of rats on the experimental (fish protein) diet*

Rat no.	1st lactation: No. of pups Original ♂	Original ♀	Weaned ♂	Weaned ♀	Av. wt. at weaning ♂	Av. wt. at weaning ♀	Gain or loss in mother's wt. during lactation	2nd lactation: No. of pups Original ♂	Original ♀	Weaned ♂	Weaned ♀	Av. wt. at weaning ♂	Av. wt. at weaning ♀	Gain or loss in mother's wt. during lactation
6041	3	2 (+3)	3	2 (+1)	42·7	41·3	+15	4	4	3	4	45·6	42·2	+20
6056	5	3	0	0	—	—	—	4	4	3	4	41·0	40·2	− 8
6065	2	6	2	3	56·0	53·3	+ 8	4	4	4	4	44·2	41·7	− 9
6068	4	4	0	0	—	—	—	4	4	0	0	—	—	—
6081	2	3	2	3	46·0	44·6	+10	5	3	2	1	29·0*	37·0	+12
6084	4	4	2	4	47·5	43·5	− 2	3	5	3	5	36·0	34·6	−14
6099	8	0	0	0	—	—	—	Some still-born				—	—	—
6119	4	4	2	3	44·5	43·0	+28	3	5	3	5	37·3	36·0	+12
6142	4	4	4	4	41·2	39·5	+20	4	4	4	4	42·2	36·5	+11
6143	5	3	5	3	44·6	42·0	− 9	3	5	2	5	46·5	43·0	+26
6153	4	4	0	0	—	—	—	3 (+1)	3 (+1)	0	0	—	—	—
6159	4	4	4	3	37·5	30·3	+20	4	4	0	0	—	—	—
6162	4	4	0	0	—	—	—	5	3	0	0	—	—	—
6163	4	4	4	4	40·0	40·0	+14	4	4	0	0	—	—	—
6173	4	4	4	4	44·2	44·2	− 4	5	1 (+2)	2	1 (+2)	46·0	48·0	+ 4
6174	1	7	1	6	40·0	42·1	+12	4	4	4	3	44·7	44·3	+ 9
6183	5	3	0	0	—	—	—	4	3	0	0	—	—	—
6189	2	6	0	0	—	—	—	1	2	1	2	50·0	53·0	− 6
6190	0 (+3)	1 (+2)	0	0	—	—	—	Not pregnant						
6197	4	4	2	3	51·5	45·6	+34	4	4	4	4	41·5	41·0	+ 2
6212	0	0 (+7)	0	0	—	—	—	Ate pups at birth						
6213	4	4	0	0	—	—	—	4	4	4	3	35·5	38·6	− 2
6221	4	4	4	4	38·7	36·0	+30	4	0 (+4)	4	0 (+4)	46·2	36·3	+12
6224	4	4	0	0	—	—	—	2	0	0	0	—	—	—
6236	4	4	3	2	42·6	44·0	+22	4	3	4	3	40·5	39·6	+13
6237	5	3	0	0	—	—	—	5	3	3	3	51·6	46·0	+14
Average	3·7	4·0	1·6	1·9	44·1	42·1	+14·1	3·8	3·6	2·2	2·5	42·4	41·1	+ 6·0
% of pups weaned			43·3	47·6						56·8	68·7			
			45·0							62·8				
% of does lactating successfully:			54						64					

N.B. In the "no. of pups" column + indicates substituted offspring.
* One ♂ weighed at weaning only 20 g.

two reproductive cycles on the basal diet alone. Two consecutive matings were carried out in March and June 1937 and the results are given in Table II. The methods were as previously described.

This time again the results were not encouraging, the number of successful lactations being too high. Thus, 54% of the females at the first mating and 64% at the second mating reared young and 45 and 63% of the available young were weaned. Though the percentage of successful lactations in this experiment was a little lower than that usual in our stock colony (see Table IV below), the percentage of lactational failures was very much lower than that reported by Nakahara & Inukai [1933]. For reasons more fully set out in the discussion we do not believe that this basal diet could be used by us satisfactorily in the study of specific lactational factors.

Experiment involving hormonal treatment

An interesting point which emerges not only from this work but also from our stock colony records has been investigated further. We find, namely, that a female which fails to raise her young after her first parturition is more likely to fail after subsequent ones than a female which raises her first litter.

For example, in the first experiment (Table I) 7 rats failed twice, while out of 26 which raised first litters and produced second litters 21 weaned them successfully. Again, in the second experiment (Table II) 7 rats failed twice, but out of 14 which suckled once 13 were successful for the second time. It should be added, however, that a successful lactation may quite well follow an unsuccessful one, as for example in the second experiment where 4 rats reared pups from a second pregnancy, having failed to do so from the first one. Conversely, 2 rats which nursed their first litter normally failed to bring up the second. Such individual fluctuations in the lactational performance when the diet apparently remains unchanged have led us to suspect that the failure to rear young under normal (stock colony) conditions may be due to failure at some point of the hormonal mechanism now known to be involved in lactation [cf. Nelson, 1936; Folley, 1938]. In view of the importance of the anterior pituitary in lactation, it is reasonable to suspect that such failures might be due to lack of an anterior pituitary hormone. An attempt to explore this possibility was therefore made. As already mentioned, a certain proportion of the rats belonging to our stock colony fail to rear their litters. 21 such females, all of which had experienced their first parturition but had failed to rear their young, were used for an experiment on the possible connexion between lactational failures and the anterior pituitary hormones. In most cases living young were born to these females but all had died within a few days of birth. In three cases, marked with an asterisk in Table III, the young, as far as could be ascertained, were born dead, so that it is not really certain that the mother could not have reared them had they been alive.

The rats were divided at random into two groups and one group of 11 received on the day after their second parturition a subcutaneous implantation of one compressed tablet weighing about 80 mg. of acetone-dried and powdered pig anterior pituitary. The remaining 10 females were given no treatment.

The litters were treated at birth as already described on p. 1992 and the surviving young were weaned at 21 days. The results of the experiment are presented in Table III, from which it is evident that on this occasion the treated rats lactated no better than the controls. The marked incidence (over 75%) of failures confirms our view that female rats which fail once to rear their young are likely to fail again, but the experiment throws no light on the cause of the failures.

Table III. *Effect of one treatment with anterior pituitary powder on the ability to rear their second litters of rats which failed to rear their first litters on the stock diet*

	No. of pups								
	Born		Substitutes		Weaned		Av. wt. at weaning		Gain or loss in mother's wt. during lactation
Rat no.	♂	♀	♂	♀	♂	♀	♂	♀	
Treated rats									
6390	5	5	—	—	4	4	41·7	42·5	+22
6480	1	3	—	—	1	3	52·0	48·0	−20
6562	4	6	—	—	0	0	—	—	—
6460	1	7	—	—	0	0	—	—	—
6438*	1	2	—	—	0	0	—	—	—
6463*	Some still-born		2	4	0	0	—	—	—
6527	1	1	—	2	0	0	—	—	—
6362*	1	0	—	5	0	0	—	—	—
6410	Some still-born		2	2	0	0	—	—	—
6590	4	4	—	—	0	0	—	—	—
6499	4	3	—	—	0	0	—	—	—
Av.	2·9	4·9	—	—	0·6	0·8	46·9	45·3	+ 1
% of pups weaned					19·2	15·9			
					17·5				
% of does lactating successfully:					18				
Control rats									
6513	8	5	—	—	7	0	43·5	—	+27
6420	6	2	—	—	4	2	49·5	47·5	+ 3
6422	0	3	4	—	0	0	—	—	—
6391	5	1	—	—	0	0	—	—	—
6579	Some still-born		3	3	0	0	—	—	—
6432	2	3	—	—	0	3	—	59·0	+ 4
6455	2	3	1	1	0	0	—	—	—
6447	2	5	—	—	0	0	—	—	—
6596	5	7	—	—	0	0	—	—	—
6445	6	1	—	1	0	0	—	—	—
Average	4·9	3·9	—	—	1·2	0·6	46·5	53·3	+11·3
% of pups weaned					25·0	14·3			
					19·6				
% of does lactating successfully:					30				

* Only dead pups in first pregnancy.
N.B. Average of "pups born" includes substituted offspring.

Lactational performance of stock colony

The stock diet, the composition of which is given on p. 1989, was introduced into the laboratory in 1931 as the best of several diets tried and has been used ever since. This diet, containing a generous allowance of fresh liver, gives excellent fertility and growth. Nevertheless, a certain percentage of stock colony females fail to rear their young. A record of such failures for the years 1932–8 is set out in Table IV. It is hoped to present later a more detailed statistical analysis of the extensive data which we possess relating to the lactational performance of our stock colony rats. It will be seen that while in 1932 and 1933 some 90% of does weaned young, this percentage has been about 70% since 1934. The better performance in the first 2 years is possibly connected with more intensive breeding during that period, when the females were allowed only 2 weeks rest between weaning of young and next mating. Females are normally first mated at 3 months old and on those occasions when they were mated later

Table IV. *Record of litters weaned and of litters lost in the stock colony for the years 1932–8*

+ denotes weaned litters; – denotes that no pups were weaned

	1932		1933		1934		1935		1936		1937		1938		% of failures 1932–8	Total litters	% of failures 1934–8	Total litters
	+	–	+	–	+	–	+	–	+	–	+	–	+	–				
Jan.	19	0	20	3	29	22	6	7	11	5	—	—	45	30	34·01	197	41·29	155
Feb.	10	2	25	3	8	8	—	—	2	1	12	14	—	1	33·72	86	52·17	46
March	16	1	5	2	11	8	24	12	2	2	41	10	10	16	31·88	160	35·30	136
April	17	1	38	1	1	8	1	0	22	7	6	5	31	6	19·44	144	29·89	87
May	12	4	46	3	8	7	—	—	29	4	18	7	56	17	19·91	211	15·75	146
June	21	2	—	—	31	7	29	8	—	—	30	8	11	0	17·01	147	18·55	124
July	10	5	21	1	4	0	4	6	—	—	—	—	0	4	29·09	55	55·56	18
Aug.	34	2	20	2	—	—	12	6	30	8	—	—	—	—	15·79	114	25·00	56
Sept.	8	1	31	8	32	5	11	4	3	0	73	36	—	—	25·47	212	27·44	164
Oct.	18	2	2	3	2	0	—	—	14	11	—	—	—	—	30·77	52	40·74	27
Nov.	1	2	—	—	21	7	2	1	—	—	—	—	—	—	29·41	34	25·81	31
Dec.	31	4	7	4	6	0	9	4	28	9	29	11	—	—	22·54	142	25·00	96
Totals	197	26	215	30	153	72	98	48	141	47	209	91	153	74	—	1554	—	1086
% of failures	11·66		12·77		32·00		32·88		25·00		30·33		32·60					

The high percentage of failures in Jan./Feb. 1934, Sept. 1937 and Jan. 1938 may possibly be due to the fact that the majority of rats used were 5 months old when first mated. Further, since 1933 the does have had longer rests between litters.

(Jan.-Feb. 1934, Sept. 1937 and Jan. 1938) there was a high percentage of failures.

A comparison of stock colony females and of the animals on the "synthetic" diets (Tables I, II and IV) shows that there is little difference between them regarding lactational performance if the criterion of successful lactation is the ability to rear young. Whether or not the synthetic diets used by us would prove in the long run adequate for growth and reproduction is a matter of conjecture. What remains certain is that the simplified diets satisfied, in these experiments at least, the lactational requirements of the nursing mothers almost as well as a stock diet of natural foodstuffs. It is significant that the stock diet supplies to lactating mothers over 1 g. of fresh liver daily and that the addition of such liver to the simplified diet was without effect.

The secretion of milk by rats which fail to rear litters

When litters are examined and weighed after birth it is our custom to see whether by gentle massage milk can be expressed from the mother's nipples. We have almost invariably been able to obtain a drop or two of milk in this way irrespective of whether the mother failed later to feed and rear her litter or reared it successfully.

Discussion

In considering the question of dietary requirements for lactation, specific dietary lactational factors (if such exist) should be carefully distinguished from dietary constituents which are essential whether or not the animal is lactating and the requirements for which may be greatly increased during lactation. Specific dietary lactational factors may be defined as dietary constituents which are essential for lactation alone of physiological processes. In order to prove the existence of such specific "lactation vitamins" the first need, as Nakahara & Inukai [1933] recognized, is to discover a diet which, while allowing satisfactory growth and reproduction, causes complete failure of lactation without any other pathological effects. Our experiments, contrary to the findings of the Nakahara school, fail to provide any evidence of the existence of such dietary factors, since, on a diet which was as far as we could make it similar to the "Factor L"-deficient diet of the Japanese workers, a satisfactory proportion of our rats was able to suckle and wean two successive litters. It is of course possible that our diet might not prove so satisfactory over a longer period and it must further be borne in mind in connexion with our failure to confirm the results of Nakahara & Inukai [1933] that diets of the same gross composition often give varying results in different laboratories. Nakahara *et al.* [1938] have recently reported that rats experiencing their second lactations on the "Factor L"-deficient diet after having lactated once on a normal diet do not fail so signally to lactate as rats undergoing their first lactation on the "Factor L"-deficient diet. This would not explain our failure to confirm their original results, however, since we used rats experiencing their first lactation.

Incidentally, it is of interest to note that our experimental diet gave satisfactory growth, since the post-weaning growth rate of a group of rats fed solely on our version of the Japanese diet was as good as that of our stock colony rats (see Fig. 1).

The failure to rear their young which, as we have seen, is normally exhibited by about 30% of our stock colony mothers, is probably not due to lack of specific dietary lactational factors, since milk can almost invariably be expressed from the teats of such rats. Nakahara & Inukai [1933; 1934], on the other hand,

reported that the mammae of rats which had failed to lactate on their "Factor L" deficient diet usually showed no signs of milk secretion on histological examination. We have not yet carried out histological studies on the

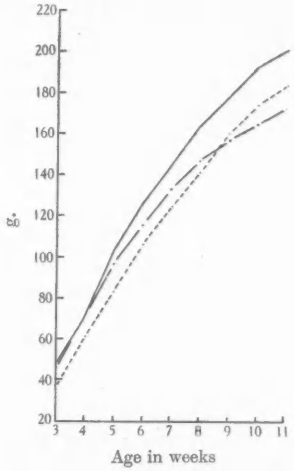

Fig. 1. Growth of female rats on the stock colony diet ·—· and ····· and on the experimental (fish protein) diet ——.—— Fish protein diet, December 1936. ·—· Stock diet, August 1936. ····· Stock diet, March 1937.

mammary glands of our stock colony mothers which fail to rear their young, but we believe that in such cases the failure to wean young may possibly be due to factors of a psychological nature. It is evident that failure to rear young may be due to other causes than lack of milk secretion. Thus Hain [1935] attributed the death of the young of oestrogen-treated rats to the unwillingness of the mother to allow the young to suckle rather than to inhibition of milk formation, while Daniels & Everson [1935] found that a high percentage of the young of rats fed on diets deficient in manganese died because they were too weak to suckle.

As far as they go, our experiments support the findings of Cox & Imboden [1936, 1, 2] that rats can lactate normally on simplified diets and we have not obtained any evidence of the existence of specific dietary factors stimulating lactation qualitatively different from the dietary principles normally required by the non-lactating rat.

Summary

1. A simplified diet of ground rice, casein, butter, salts and yeast was found to produce satisfactory lactation in rats. Additions of fresh liver, liver extract or yeast were without effect on the lactational performance.

2. Fairly satisfactory lactation was also obtained on a similar diet in which casein was replaced by fish protein.

3. Stock colony rats which failed to rear their first litters were found to be more likely to lose their second litters than rats which successfully raised their first litters. Subcutaneous implantation of tablets of pig anterior pituitary into such rats just after the second parturition had no effect on their ability to rear their young.

Note added 29 October 1938. In view of the findings reported on p. 1992 concerning the effect of additional yeast it is of interest that Perla [1937] believes that an excess of vitamin B_1 has an untoward effect on lactation and the nursing instinct of rats.

REFERENCES

Coward (1938). Biological Standardisation of the Vitamins. (London: Baillère, Tindall and Cox, p. 8.)
Cox & Imboden (1936, 1). *Proc. Soc. exp. Biol., N.Y.*, **34**, 443.
—— —— (1936, 2). *J. Nutrit.* **11**, 147.
Daniels & Everson (1935). *J. Nutrit.* **9**, 191.
Dragstedt, van Prohaska & Harms (1936). *Amer. J. Physiol.* **117**, 175.
Feaster & Nelson (1935). *Amer. J. Physiol.* **115**, 147.
Folley (1938). *Lancet*, **235**, 389.
Hain (1935). *Quart. J. exp. Physiol.* **25**, 303.
Kon (1931). *Lancet*, **220**, 778.
—— & Booth (1934). *Biochem. J.* **28**, 121.
—— & Peskett (1933). Unpublished results.
Mapson (1932). *Biochem. J.* **26**, 970.
—— (1933). *Biochem. J.* **27**, 1061.
Meyer (1936). *Rep. Ill. agric. Exp. Sta.* 1934–5, **48**, 289.
Nakahara & Inukai (1933). *Sci. Pap. Inst. phys. chem. Res., Tokyo*, **22**, 301.
—— —— (1934). *Sci. Pap. Inst. phys. chem. Res., Tokyo*, **24**, 33.
—— —— & Kato (1934). *Sci. Pap. Inst. phys. chem. Res., Tokyo*, **24**, 155.
—— —— —— & Ugami (1936). *Sci. Pap. Inst. phys. chem. Res., Tokyo*, **29**, 47.
—— —— & Ugami (1935). *Sci. Pap. Inst. phys. chem. Res., Tokyo*, **28**, 152.
—— —— —— (1937). *Sci. Pap. Inst. phys. chem. Res., Tokyo*, **31**, 42.
—— —— —— (1938). *Sci. Pap. Inst. phys. chem. Res., Tokyo*, **34**, 250.
Nelson (1936). *Physiol. Rev.* **16**, 488.
Outhouse (1937). *Rep. Ill. agric. Exp. Sta.* 1935–6, **49**, 285.
Perla (1937). *Proc. Soc. exp. Biol. N.Y.* **37**, 169.
Richter, Holt & Barelare (1938). *Amer. J. Physiol.* **122**, 734.
Smith & Anderson (1929). *Science*, **70**, 98.
Smith & Seagers (1934, 1). *J. Nutrit.* **7**, 195.
—— —— (1934, 2). *J. Nutrit.* **7**, 209.
Swanson & Nelson (1937). *Rep. Ia agric Exp. Sta.* 164.
Tso (1927). *Proc. Soc. exp. Biol., N.Y.*, **24**, 465.
Wilkinson & Nelson (1931). *Amer. J. Physiol.* **96**, 139.

CCLIX. THE REDUCTION OF NITRATE TO AMMONIA BY *CLOSTRIDIUM WELCHII*

By DONALD DEVEREUX WOODS[1]

From the Biochemical Laboratory, Cambridge

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Many bacteria are able to reduce nitrate to nitrite and this reaction has long been used in the identification and classification of these organisms. Quastel *et al.* [1925] showed that the reduction took place as a result of a coupled reaction with some H donator (e.g. lactate) and it was demonstrated that certain facultative anaerobes could grow anaerobically in the presence of nitrate on media which, in the absence of nitrate, could only support aerobic growth. The nitrate replaces oxygen as the final H acceptor [Quastel & Stephenson, 1925]. Green *et al.* [1934], working with toluene-treated *Bact. coli* and formate and lactate as H donators, concluded that the H transfer to nitrate takes place via a carrier; the nature of the natural carrier is so far unknown. Organisms possessing the hydrogenase enzyme [Stephenson & Stickland, 1931] as well as the nitrate-activating enzyme can utilize molecular H_2 as reducing agent.

Stickland [1931] made a detailed study of the reduction of nitrates by suspensions of *Bact. coli*. With lactate or molecular hydrogen as donator nitrate was reduced quantitatively to nitrite. With toluene-treated cells the H_2 uptake was also theoretical. There was no indication of any further reduction of nitrite with either plain or toluene-treated cells.

Reduction of nitrate to NH_3 has been obtained by Stocklasa [1908] with *Azotobacter chroococcum* and *radiobacter* and by Stocklasa & Vítek [1905] with four other organisms. In growth experiments, with *azotobacter* and *radiobacter* on an inorganic medium plus mannitol and nitrate, there was a disappearance of nitrate and formation of nitrite and NH_3 both aerobically and anaerobically. The effect was most marked in the case of *radiobacter* where, after 20 days, the inorganic N fraction consisted solely of NH_3; there was also considerable denitrification. With washed suspensions of *Bact. coli* and nitrate Aubel *et al.* [1937] found a small production of NH_3 in addition to some nitrite, when glucose was used as H donator.

In the course of a general investigation of the enzymic make up of washed cells of *Cl. welchii*, hydrogenase was detected and nitrate was found to be reduced in the presence of H_2. The H_2 uptake was greatly in excess of that required for the formation of nitrite. The work described in the present paper shows that nitrate, nitrite and hydroxylamine are reduced quantitatively to NH_3 by H_2 in the presence of washed suspensions of *Cl. welchii*. Similar results have been obtained with one strain of *Bact. coli*. Whilst this work was in progress a further paper by Aubel [1938] appeared, in which the quantitative reduction of nitrite to ammonia by suspensions of *Bact. coli* with glucose as H donator was demonstrated.

[1] Beit Memorial Research Fellow.

EXPERIMENTAL

Methods of estimation

(1) *Hydrogen.* H_2 uptakes were measured in Warburg manometers. CO_2 absorbers were placed in the inner tubes as the bacterial suspensions produced a little CO_2 when incubated. The main cup usually contained buffer and bacterial suspension and the substrate was tipped in from a side bulb after equilibration. The manometers were filled with H_2 or N_2 purified by passage over heated copper.

(2) *Ammonia.* NH_3 was determined on 1 or 2 ml. samples from the manometer vessels by the Conway & Byrne [1933] technique followed by nesslerization. Since NH_2OH ($\equiv 5\,\mu$g. N) produces a yellow opalescence and finally a dark precipitate in Nessler's reagent it was necessary to ascertain whether any distillation of NH_2OH occurred in the Conway apparatus. After distilling 56 μg. NH_2OH-N for 3 hr. at 37° the distillate gave no trace of reaction with Nessler's reagent, whilst 56 μg. NH_3-N were distilled quantitatively in 1 hr. at 37°.

(3) *Nitrite.* Nitrite was estimated colorimetrically by the Griess-Ilosvay reagent as described by Stickland [1931]. Standard solutions of nitrite were checked by this method or volumetrically with $KMnO_4$.

(4) *Hydroxylamine.* Tests for NH_2OH were made and standard solutions checked by oxidation with I_2 to NO_2 and estimation of the latter colorimetrically [Endres & Kaufman, 1937].

The dry wt. of bacterial suspensions was estimated by means of a photoelectric turbidimeter [Clifton *et al.* 1935].

Unless otherwise stated all experiments were carried out at 37° and in phosphate buffer *p*H 7·1 of final concentration 0·05–0·1 *M*. To facilitate comparison quantitative data are all expressed in μl., making the assumption, in the case of solids and liquids, that 1 g. mol. $\equiv$ 22·4 l. Thus 1 μg. NH_3-N, NO_3-N, NO_2-N or NH_2OH-N $\equiv 1{\cdot}6\,\mu$l. KNO_3 (B.D.H. "Analar") and $NaNO_2$ (Kahlbaum *pro analyse*) were used in this work. Hydroxylamine solutions were freshly prepared daily from the pure hydrochloride (Fraenkel and Landau) and neutralized to *p*H 7·1.

Growth of organisms

The strain of *Cl. welchii* used in this work was that of the National Collection of Type Cultures No. 273 *Bacillus welchii S.R. 9* and isolated by Robertson from a fatal case of gas gangrene in 1914. Cultures of this organism have a tendency to "rope" and to form rough colonies; the preparation of suspensions is then difficult. The following procedure (Robertson, personal communication) was adopted for the maintenance of stock cultures. The organism was plated anaerobically on tryptic caseinogen broth-agar, a smooth colony picked off, and, after a few rapid subcultivations through Robertson's meat medium, sown into a number of tubes of alkaline egg medium. The latter were incubated 48 hr. anaerobically, sealed and stored in the dark at room temp. A working stock culture for sowing bulk cultures was maintained by daily subcultivation from one tube of Robertson's meat to another and incubation for 10–12 hr. anaerobically. After 7–12 such subcultivations the culture usually "roped" and a new subculture from the stock on alkaline egg was taken. Every 4–6 weeks the organism was replated and a new stock of alkaline egg cultures put up.

For the preparation of suspensions 900 ml. tryptic caseinogen digest broth *p*H 7·5, containing a few pieces of meat from Robertson's meat medium, were autoclaved, cooled and sown at once with 1 ml. fluid from a 10 hr. culture on Robertson's meat medium. The culture was incubated at 37° in a McIntosh and

Fildes anaerobic jar for 10–12 hr. The following dry wt. determinations show that the growth obtained under these conditions is fairly uniform:

No. determinations	24
Mg. dry wt. organism/ml. culture:	
Range	0·15–0·27
Mean	0·21
Standard deviation	0·035

Longer incubation does not improve growth and the suspensions are less active. The culture was centrifuged, and the cells washed twice on the centrifuge with $M/50$ phosphate, pH 7·1; for washing the concentration of the organism should not exceed 1 mg. dry wt./ml., otherwise sedimentation is not sharp. The cells were finally suspended in water or buffer to give a concentration of *ca.* 10 mg. dry wt./ml. Addition of glucose to the culture medium gives even more rapid growth and greater bulk of organisms, but production of acid is very rapid and difficult to control and suspensions were less active from the point of view of the work in the present paper.

Two strains of *Bact. coli* have also been used: (*a*) *Escherichia coli* N.T.C. No. 86 (Strain I) and (*b*) a strain originating from the Bacteriological Department of Sheffield University (Strain II). Both were grown aerobically in flasks of tryptic caseinogen broth for 16 hr. at 37° and suspensions prepared in the usual way.

Experiments with *Cl. welchii*

The reduction of nitrate

The course of the H_2 uptake by suspensions of *Cl. welchii* in the presence of nitrate is shown in Fig. 1. There is a small blank H_2 output (rarely exceeding

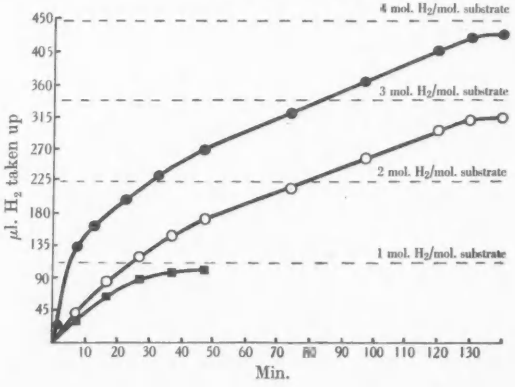

Fig. 1. Course of H_2 uptake. • nitrate, ○ nitrite, ■ hydroxylamine. 1 ml. bacterial suspension (6·2 mg./ml.), 1 ml. $M/5$ phosphate buffer pH 7·1, 0·1 ml. $M/20$ substrate, 0·4 ml. water.

15 μl./hr.) with the organisms alone; a correction for this has been made. It will be seen that there is an initial rapid uptake of H_2 equivalent to about 1 mol. per mol. NO_3 followed by a slower uptake which continues until approx. 4 mol.

H_2 have been absorbed and then ceases abruptly. The reduction of nitrate to nitrite requires only 1 mol. H_2:

$$HNO_3 + H_2 = HNO_2 + H_2O, \qquad \text{.....(1)}$$

whilst an uptake of 4 mol. H_2 would correspond to a complete reduction to NH_3:

$$HNO_3 + 4H_2 = NH_3 + 3H_2O. \qquad \text{.....(2)}$$

The presence of a volatile alkali giving the reaction with Nessler's reagent characteristic of NH_3 is readily demonstrated at the end of such an experiment. A number of experiments in which both H_2 uptake and NH_3 formation were estimated quantitatively have been carried out and the results of three such experiments are summarized in Table I. Controls were put up as follows: (*a*) without NO_3, (*b*) without H_2 (gas phase N_2), (*c*) without organisms. The quantitative data for the complete system NO_3-H_2-organism are in close agreement with the requirements of (2). The "H_2 uptake" in the N_2 gas phase experiments is due to the fact that there is a small H_2 evolution by the organisms alone in N_2 which is partially or completely suppressed in the presence of nitrate. It will be seen that there is also a small NH_3 production from nitrate in N_2; this production is always more than can be accounted for by the blank H_2 uptake in N_2 assuming that 4 mol. H_2 are required for formation of 1 mol. NH_3 (see last column $\frac{\mu l.\ H_2}{4}$ and cf. $\mu l.$ NH_3 found). It would seem, therefore, that some unknown H donators, apart from H_2, are present in the cell which can also reduce NO_3 to NH_3. It will be shown later that this effect is more marked in the case of NO_2 and NH_2OH.

Table I

Theoretical values are those required by equation (2). The organism blank has been corrected for where necessary. 1 ml. bacterial suspension (*ca.* 10 mg./ml.), 1 ml. *M*/5 phosphate buffer *p*H 7·1, 0·1 ml. *M*/20 or 0·2 ml. *M*/50 KNO_3, water to 2·5 ml.

System	Exp. no.	Gas phase	H_2 uptake ($\mu l.$) Found	Calc.	% calc	NH_3 production ($\mu l.$) Found	Calc.	% calc.	$\mu l.$ H_2 found / 4
$NO_3 + H_2 +$ *Cl. welchii*	1	H_2	330	358	92	91	89·5	102	83
	2	H_2	443	448	99	115	112	102	111
	3	H_2	323	358	90	84	89·5	94	81
$NO_3 +$ *Cl. welchii*	1	N_2	9	358	2·5	6	89·5	6·8	2
	2	N_2	4	448	1·0	6·5	112	5·9	1
	3	N_2	26	358	7·3	9	89·5	10·0	7
$NO_3 + H_2$	1	H_2	0	358	0	0	89·5	0	—

Table II summarizes the results of a number of experiments on the quantitative relationship between H_2 uptake, NH_3 production and original NO_3 and shows close agreement with equation (2).

Table II

All values corrected for organism blank

	mol. H_2 per mol. NO_3	mol. NH_3 per mol. NO_3	mol. H_2 per mol. NH_3
No. of determinations	18	11	11
Range	3·58–3·96	0·89–1·03	3·52–4·29
Mean	3·75	0·98	3·81
Standard deviation	0·111	0·057	0·224
Theory (equation 2)	4·0	1·0	4·0

The reduction of nitrite

The initial rapid uptake of 1 mol. H_2 that occurs in the reduction of NO_3 (see Fig. 1) suggested that reduction of NO_3 to NO_2 might be the first stage in the production of NH_3 from NO_3. In this case NO_2 should also be reduced when incubated with suspensions of *Cl. welchii* in presence of H_2. This was found to be the case; a steady H_2 uptake reaching almost 3 mol. H_2 per mol. NO_2 being obtained (see Fig. 1). This would correspond to the final reduction of NO_2 to NH_3:

$$HNO_2+3H_2=NH_3+2H_2O. \qquad \text{.....(3)}$$

NH_3 was again found to be present and Table III gives the results of three quantitative experiments. The data for the complete system are in good agreement with equation (3). As with NO_3 the production of NH_3 in N_2 is greater than can be accounted for by the H_2 uptake in N_2. The effect is more marked than with NO_3 owing to the larger NH_3 production in N_2.

Table III

Theoretical values are those required by equation (3). The organism blank has been allowed for where necessary. 1 ml. bacterial suspension (*ca.* 10 mg./ml.), 1 ml. *M*/5 phosphate buffer *p*H 7·1, 0·1 ml. *M*/20 or 0·2 ml. *M*/50 $NaNO_2$, water to 2·5 ml.

System	Exp. no.	Gas phase	H_2 uptake (μl.) Found	Calc.	% calc.	NH_3 production (μl.) Found	Calc.	% calc.	μl. H_2 found / 3
$NO_2+H_2+Cl.$ *welchii*	1	H_2	299	336	89	110	112	98	100
	2	H_2	255	269	95	85	89·5	95	85
	3	H_2	246	269	92	92	89·5	103	82
$NO_2+Cl.$ *welchii*	1	N_2	3	336	0·9	12	112	10·4	1
	2	N_2	6	269	2·2	7	89·5	8·0	2
	3	N_2	41	269	15·2	33	89·5	37·0	14
NO_2+H_2	1	H_2	0	336	0	0	112	0	—

A number of experiments in which the quantitative relations between the various reactants of the complete system were determined are summarized in Table IV. The close agreement with equation (3) is apparent.

Table IV

All values corrected for organism blank

	mol. H_2 per mol. NO_2	mol. NH_3 per mol. NO_2	mol. H_2 per mol. NH_3
No. of determinations	11	6	6
Range	2·53–2·86	0·95–1·03	2·49–3·0
Mean	2·70	0·99	2·69
Standard deviation	0·112	0·036	0·193
Theory (equation 3)	3·0	1·0	3·0

The reduction of hydroxylamine

The possibility that NH_2OH might be an intermediate in the reduction of NO_3 and NO_2 to NH_3 was at once apparent.

$$HNO_3+3H_2=NH_2OH+2H_2O \qquad \text{.....(4)}$$
$$HNO_2+2H_2=NH_2OH+H_2O \qquad \text{.....(5)}$$
$$NH_2OH+H_2=NH_3+H_2O. \qquad \text{.....(6)}$$

It was found that NH_2OH was rapidly reduced with an uptake of approx. 1 mol. H_2 per mol. NH_2OH (see Fig. 1), and NH_3 was found present at the end of the

experiment. The results of three quantitative experiments are given in Table V and are in good agreement with (6). The production of NH_3 in N_2 is larger than with either NO_3 or NO_2 and again this NH_3 formation cannot be accounted for by the H_2 uptake in N_2 (see columns 3 and 6). The greater magnitude of the effect in this case may be due to the same amount of endogenous H donators being able to reduce 3 or 4 times as much NH_2OH as NO_2 or NO_3 respectively; alternatively, these donators may be able to reduce NH_2OH more rapidly than the other substrates. The almost quantitative H_2 uptake shows that the reduction by H_2 takes place preferentially to the reduction by cell donators.

Table V

Theoretical values are those required by equation (6). The organism blank has been allowed for where necessary. 1 ml. bacterial suspension (*ca.* 10 mg./ml.), 1 ml. *M*/5 phosphate buffer *p*H 7·1, 0·1 ml. *M*/20 or 0·2 ml. *M*/50 NH_2OH, water to 2·5 ml.

System	Exp. no.	Gas phase	H_2 uptake (μl.) Found	Calc.	% calc.	NH_3 production (μl.) Found	Calc.	% calc.
$NH_2OH + H_2$ + *Cl. welchii*	1	H_2	77	89·5	86	85	89·5	95·5
	2	H_2	75	89·5	84	91	89·5	101
	3	H_2	96	112	86	106	112	96
NH_2OH + *Cl. welchii*	1	N_2	2	89·5	2·2	18·5	89·5	20·7
	2	N_2	6	89·5	6·7	37	89·5	40·8
	3	N_2	4	112	3·6	25	112	22·7
$NH_2OH + H_2$	1	H_2	0	89·5	0	0	89·5	0

The quantitative relationships between the initial and final products of the reaction in a number of determinations are summarized in Table VI and agree fairly well with equation (6).

Table VI

All values corrected for organism blank

	mol. H_2 per mol. NH_2OH	mol. NH_2 per mol. NH_2OH	mol. H_2 per mol. NH_2OH
No. of determinations	13	6	6
Range	0·81–0·95	0·85–1·01	0·83–1·10
Mean	0·88	0·94	0·92
Standard deviation	0·046	0·054	0·094
Theory (equation 6)	1·0	1·0	1·0

Evidence that nitrite is an intermediate in the reduction of nitrate

The initial rapid uptake of about 1 mol. H_2 during the reduction of NO_3 suggests that there is a preliminary formation of NO_2 which is then further reduced. It is significant that, after the initial burst, the succeeding slower rate of H_2 uptake is equal, within experimental error, to the rate of H_2 uptake with NO_2 (Fig. 1); this result has always been obtained in experiments in which the same batch of organisms has been used with NO_3 and NO_2.

The comparative rates of NH_3 production from NO_3 and NO_2 provide further evidence that NO_2 is an intermediate. The rate of H_2 uptake with NO_2 is slower than the initial rate with NO_3; taking the figures for the first 7 min. of the experiment of Fig. 1 and dividing by 3 or 4 to correct for the difference in total H_2 uptake the resulting figures give a measure of the maximum possible rate of NH_3 formation:

	μl. H_2	μl. $H_2/4$	μl. $H_2/3$
NO_3	133	33	—
NO_2	40	—	13

If NO_2 is an intermediate the slower reduction of NO_2 should control the overall rate of NH_3 production from NO_3 and the rates of NH_3 production from the two substances should be equal. Fig. 2 shows that this is the case. For these determinations a series of manometers were set up containing NO_3, NO_2 or NH_2OH and bacterial suspension in a gas phase of H_2. The course of the reaction was followed by the H_2 uptake and at intervals manometers were rapidly disconnected, the cups plunged into ice-salt mixture to stop the reaction and NH_3 estimated on 2 ml. samples. Controls without substrate were carried out.

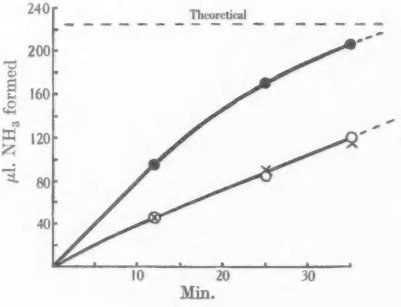

Fig. 2.

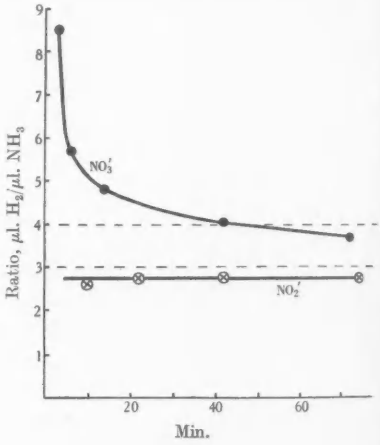

Fig. 3.

Fig. 2. Course of NH_3 production. × nitrate, o nitrite, • hydroxylamine. 1 ml. bacterial suspension (7·1 mg./ml.), 1 ml. *M*/5 phosphate buffer *p*H 7·1, 0·2 ml. *M*/20 substrate (224 μl.), 0·3 ml. water.

Fig. 3. Comparison of H_2 uptake with NH_3 production. 1 ml. bacterial suspension (10 mg./ml.), 1 ml. *M*/5 phosphate buffer *p*H 7·1, 0·2 ml. *M*/20 NO_3 or NO_2, 0·3 ml. water.

In Fig. 3 the ratio of H_2 uptake to NH_3 formation at various stages during the reduction of NO_3 is plotted. In order to obtain a more precise value for the H_2 uptake for the early samples it was necessary to stop the reaction by tipping in 0·2 ml. 10 % H_2SO_4 from a second side bulb as rapidly as possible after reading the manometer; even so the first two points may be up to 5 % lower than the true values. It will be seen that in the early stages the H_2 uptake is in excess of the 4 mol. required to reduce NO_3 to NH_3, but soon falls to approximately this value. The curve suggests that there may be an accumulation of a rapidly formed primary product of reduction (presumably NO_2) in the early stages. Experiments were carried out to test this possibility. A series of manometers containing NO_3 and *Cl. welchii* suspension in a H_2 gas phase were set up together with controls without NO_3. The NO_3 was tipped in from a side bulb after equilibration and the uptake of H_2 followed. At frequent intervals manometers were rapidly taken down and the cups plunged into ice-salt mixture. The contents were transferred to conical centrifuge tubes which were again cooled and then centrifuged at 0° till a sharp separation of the organisms was attained. 1 ml. of the clear supernatant was pipetted off (the organisms stir up easily) and used for nitrite

determination by the Griess-Ilosvay method. It was found impossible to use trichloroacetic acid as protein precipitant as there was a 20–30 % loss of NO_2 on acid treatment of quantities of the order found in these experiments. This loss was increased if the acid (though not the neutral) solution was filtered through kieselguhr.

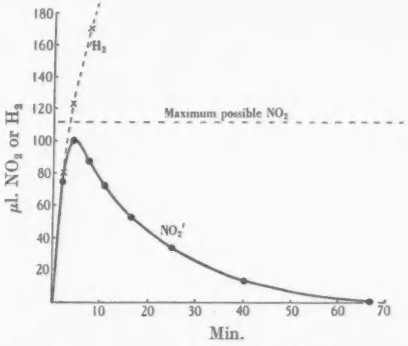

Fig. 4. Appearance and disappearance of nitrite. 1 ml. bacterial suspension (8·3 mg./ml.), 1 ml. *M*/5 phosphate buffer *p*H 7·1, 0·1 ml. *M*/20 KNO_3, 0·4 ml. water.

The curves given in Fig. 4 show the rapid production of NO_2 during the early stages and its removal as the reaction proceeds. The maximum NO_2 production reaches 89 % of that theoretically possible from the NO_3 added. During the period of rising NO_2 concentration the H_2 uptake is almost all accounted for by the NO_2 formed. Table VII shows that during the period of diminishing NO_2

Table VII

All values less organism blank

Time min.	μl. H_2 absorbed (*a*)	μl. NO_2 present	μl. H_2 to convert NO_2 into NH_3 (*b*)	(*a*) + (*b*)
2	80	75	—	—
4	123	100	300	423
7·5	170	87·5	262	432
11	211	72·5	218	429
16·5	263	53	159	422
25	321	34	102	423
40	383	13·5	40	423
70	421	0	0	421

concentration the H_2 already absorbed plus the H_2 required to reduce the NO_2 present to NH_3 agrees with the total H_2 uptake of the reaction. Thus, both qualitatively and quantitatively, these experiments show that NO_2 is formed as an intermediate product during the reduction of NO_3 by H_2.

Evidence in favour of hydroxylamine as an intermediate in the reduction of nitrite

It has already been shown that *Cl. welchii* reduces hydroxylamine to NH_3 by H_2 and the question arose as to whether it may be formed as an intermediate during the reduction of NO_2 and NO_3 (see equations (5) and (6)). If this is so it is necessary that the rate of NH_3 formation from NH_2OH should be at least as great as that from NO_3 and NO_2 with the same batch of organisms. Reference to

Fig. 2 shows that this is so, the rate in this particular experiment being twice that with NO_3 and NO_2. In nine similar experiments with different batches of organisms the ratio rate NH_3 from NH_2OH/rate of NH_3 from NO_2 varied from 1·2 to 4·6 (six values lay between 2·2 and 3·3) but was never less than 1. From this point of view therefore NH_2OH satisfies the conditions for an intermediate.

If NH_2OH is an intermediate it will be reduced to NH_3 as rapidly as it is formed and the rate of reduction of NO_2 to NH_2OH will set a limit to the overall rate of NH_3 production from NO_2; there would therefore be little possibility of a direct detection of NH_2OH during the reaction. This view is supported by data on the ratio H_2 uptake/NH_3 produced at various stages during the reduction of NO_2. Fig. 3 shows that this ratio remains constant at just below 3 so that throughout the reaction the H_2 uptake is completely accounted for by the NH_3 formed.

Attempts were therefore made by the addition of ketonic substances to trap any NH_2OH which might be formed. So far no fixative has been found which forms a sufficiently stable oxime and at the same time is not itself attacked by the organism. Diacetyl completely inhibited the reduction of NH_2OH to NH_3 and also brought about a partial inhibition of NH_3 formation from NO_3 and a corresponding partial inhibition of the H_2 uptake. The results are rendered somewhat unsatisfactory by the fact that diacetyl itself is decomposed by the organism with uptake of H_2; large corrections have therefore to be applied to the H_2 uptake figures and the possible effect on the NO_3 reaction of reduction products of diacetyl is unknown. Oxaloacetate, pyruvate and α-ketoglutarate formed insufficiently stable oximes.

Summarizing the position as regards the possibility that NH_2OH is an intermediate, it must be emphasized that whilst all the data so far obtained are in accordance with such a view, there is no conclusive evidence of its truth.

Effects of concentration of substrate, pH and age of suspension on rate of reduction of NO_3, NO_2 and NH_2OH

Concentration of substrate. Table VIII gives the Q_{H_2} values obtained for the 5 min. period immediately after tipping in the substrate. The value for NO_3 gives an approximate idea of the rate of the preliminary reduction of NO_3 to NO_2 as the period measured is within the initial burst of the H_2 uptake (Fig. 1).

Table VIII

All values corrected for organism blank. 1 ml. bacterial suspension (6·2 mg./ml.) 1 ml. *M*/5 buffer *p*H 7·1, 0·1, 0·2 or 0·5 ml. *M*/20 substrate, water to 2·5 ml.

	Initial Q_{H_2} values		
Conc. substrate	NO_3	NO_2	NH_2OH
0·01 *M*	−349	−95	−99
0·004 *M*	−339	−77	−66
0·002 *M*	−211	−49	−41

It will be realized from these figures that most of the quantitative work already described has been carried out with suboptimal concentrations (0·001–0·002 *M*) of substrate. This was done in order to be able to measure total H_2 uptakes without adding more H_2 and re-equilibrating during the reaction.

The figures of Table VIII also give some idea of the absolute activity of the organism in terms of dry weight. Q_{H_2} values of this order have always been obtained with fresh suspensions of organisms and are comparable in magnitude with Q_{O_2} values obtained with aerobic bacteria.

Age of suspension. The effect of storage in N_2 at 0° on the initial Q_{H_2} obtained with NO_3, NO_2 and NH_2OH is shown in Table IX. It will be seen that the activity is relatively stable for 10 hr., falls off seriously in 24 hr. and has almost disappeared in 46 hr. The activity falls off slightly more rapidly with NO_3 than with NO_2 and NH_2OH.

Table IX

All values less organism blank. 1 ml. bacterial suspension (8·6 mg./ml.; 1·7 mg./ml. with NO_3). 1 ml. *M*/5 buffer 7·1, 0·4 *M*/20 NO_3 or NH_2OH or 0·2 ml. *M*/20 NO_2, water to 2·5 ml.

	Nitrate		Nitrite		Hydroxylamine	
Age (hours)	Q_{H_2}	% Q_{H_2} at 2 hr.	Q_{H_2}	% Q_{H_2} at 2 hr.	Q_{H_2}	% Q_{H_2} at 2 hr.
2	−328	100	−70	100	−60	100
10	−293	90	−67	95	−57	95
24	−112	34	−30	43	−27	45
46	−4	1	−2	3	−2	3

Effect of p*H*. The *p*H curve with NH_2OH (Fig. 5) was rather unusual in that the rate of reduction increased steadily with rising *p*H throughout the range of phosphate buffer. Controls without bacterial suspension showed that there was no purely chemical reduction of NH_2OH at any *p*H used.

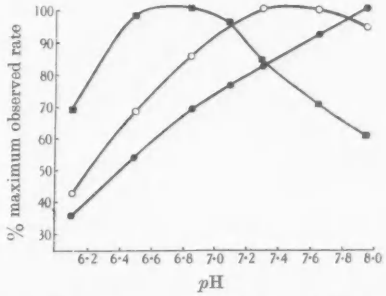

Fig. 5. Effect of *p*H. ○ nitrate, ■ nitrite, • hydroxylamine.

The relation between *p*H and rate of H_2 uptake with NO_2 is also shown in Fig. 5. The rates plotted are those for the first 5 min. period since at *p*H 6·1 and 6·5 the H_2 uptake fell off sharply and soon stopped completely (Fig. 6). It appears that the organism is rapidly inactivated by nitrite at *p*H 6·5 and below. At *p*H 7·95, although the initial rate of H_2 uptake is slower than at *p*H 6·5, there is no inactivation and the reduction proceeds to completion.

Experiments on the effect of *p*H on the initial rate of H_2 uptake with NO_3 yielded a curious result. It was found that below *p*H 7·3 there is a marked lag period of about 5 min. before the rate becomes linear (Fig. 7). This lag period is less marked or absent at *p*H 7·3 and above. This phenomenon has been observed with two other batches of organisms and no explanation can be offered for it. In the *p*H curve for NO_3 in Fig. 5 the rates are those for the second 5 min. period after tipping by which time the rate had become linear at all *p*H values. As with nitrite the reaction stops short of completion at *p*H 6·1 and 6·5, although complete inactivation took longer to occur. Thus the H_2 uptake became reduced to 1/10 of the initial value after 25 min. at *p*H 6·1 and after 35 min. at *p*H 6·5. As it

has already been shown that NO_2 is formed faster than it is removed this effect may be ascribed to the accumulation of NO_2. All the above experiments with NO_3 were carried out with 1/5 the usual concentration of organism (*ca.* 2 mg./ml.)

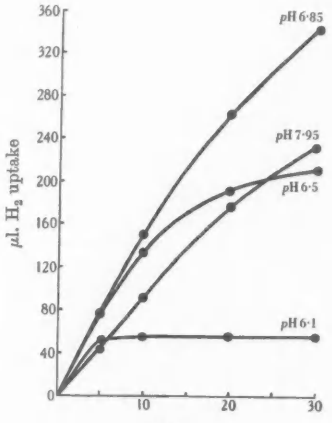

Fig. 6.

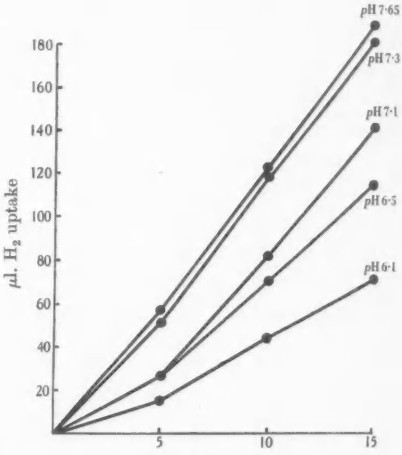

Fig. 7.

Fig. 6. Inactivation by nitrite at acid reaction. 1 ml. bacterial suspension (11·8 mg./ml.), 1 ml. *M*/5 phosphate buffer, 0·15 ml. *M*/20 $NaNO_2$, water to 2·5 ml.

Fig. 7. Lag period in H_2 uptake with nitrate. 1 ml. bacterial suspension (2 mg./ml.), 1 ml. *M*/5 phosphate buffer, 0·4 ml. *M*/20 KNO_3, water to 2·5 ml.

and a higher concentration of NO_3 (*M*/100) in order to confine the determinations to the initial rapid period during which the principal reaction is the reduction of NO_3 to NO_2.

Experiments with *Bact. coli*

Stickland [1931] has found that NO_3 is reduced quantitatively to NO_2 by H_2, in the presence of washed suspensions of *Bact. coli* (Esch.). His figures do not show any further reduction of NO_2. The chief experiments described above for *Cl. welchii* were repeated with two strains of *Bact. coli*; both reduced NO_3, NO_2 and NH_2OH to NH_3 in the presence of H_2 (Table XI), but quantitatively the rates of H_2 uptake with the various substrates varied greatly according to which strain was used (Table X). With strain II (see p. 2002) the rate of H_2 uptake, and

Table X

All values less organism blank. 1 ml. buffer *p*H 7·3, 0·1 *M*/20 substrate, 1 ml. bacterial suspension (exp. 1, 2·5 mg./ml.; exp. 2, 6·4 mg./ml.; exp. 3, 5·6 mg./ml.; exp. 4, 5·7 mg./ml.), water to 2·5 ml.

		Initial Q_{H_2}		
Strain	Exp. no.	NO_3	NO_2	NH_2OH
Strain II	1	−236	−248	−181
	2	−242	−253	−115
Strain I	1	−229	−45	−83
	2	−113	−6	−39

therefore NH_3 formation, is greater with NO_2 than with NO_3. Thus, unlike the case of *Cl. welchii*, NO_2 would be removed as fast as it is formed and the limiting factor in the rate of reduction of NO_3 to NH_3 becomes the rate of the preliminary reduction to NO_2. The rate of H_2 absorption by NH_2OH is also rapid and it follows from the data of Table X and equations (2), (3) and (6) that the rate of reduction of NH_2OH to NH_3 is even greater than that of NO_2 (as with *Cl. welchii*).

Quite different results are obtained with Strain I. Here the data resemble those for *Cl. welchii* more closely (see Tables VIII and X) except that the rate of H_2 uptake with NO_2 is slower.

Table XI

Theoretical values are those required by equations (2), (3) or (6). All values corrected for the organism blank. 1 ml. bacterial suspension (*ca.* 5 mg./ml.), 1 ml. *M*/5 phosphate buffer *p*H 7·3, 0·1 or 0·2 ml. *M*/20 substrate, water to 2·5 ml.

			H_2 uptake (μl.)			NH_3 production (μl.)			μl. H_2 found
Bact. coli	Substrate	Gas phase	Found	Calc.	% calc.	Found	Calc.	% calc.	1, 3 or 4
Strain II	NO_3	H_2	435	448	97	111	112	99	109
		N_2	1	448	0·2	0	112	0	0
	NO_2	H_2	328	336	97	114	112	102	109
		N_2	2	336	0·6	5	112	4·5	1
	NH_2OH	H_2	97	112	87	112	112	100	97
		N_2	1	112	0·9	24	112	21·3	1
Strain I	NO_3*	H_2	308	896	34·4	38	224	17	—
		N_2	14	896	1·6	14	224	6·3	—
	NO_2*	H_2	102	672	15·2	37	224	17	—
		N_2	18	672	2·7	6	224	2·6	—
	NH_2OH	H_2	97	112	87	113	112	101	97
		N_2	0	112	0	33	112	30	0

* Not taken to completion.

The quantitative data presented in Table XI show that with Strain II the H_2 uptake and NH_3 formation with NO_3, NO_2 and NH_2OH are in conformity with equations (2), (3) and (6). This is also true of Strain I with NH_2OH. Owing to the very slow reduction of NO_2 by the latter strain the experiments could not be taken to completion but the production of NH_3 and uptake of H_2 are demonstrated. Table XI also shows that with both strains NH_3 production in N_2 is similar to that obtained with *Cl. welchii* and the same possible explanations are applicable. It has not been possible to determine whether the failure of Stickland's [1931] strain to carry the reduction further than NO_2 is due to difference of strain or to different conditions of growth—both of which factors in influencing enzyme production were at that time imperfectly appreciated.

Discussion

The oxidations of NH_3 to NO_2 and NO_2 to NO_3 by the autotrophic soil organisms *nitrosomonas* and *nitrobacter* have long been known and considered as important stages in the circulation of N in nature. The reverse action—the reduction of NO_3 and NO_2 to NH_3—had until now only been demonstrated conclusively with *azotobacter* and *radiobacter* [Stocklasa, 1908] and for four other organisms [Stocklasa and Vítek, 1905]. The former are also soil organisms. The work reported in the present paper shows that such common organisms as *Cl. welchii* (one of the most widely distributed soil anaerobes) and some strains of *Bact.*

coli can also bring about this reduction energetically. It has been demonstrated that the reduction can be effected with the aid of molecular H_2; since *Cl. welchii* decomposes not only many carbohydrates but also some of the amino-acid constituents of tryptic caseinogen broth with evolution of H_2, a supply of this reducing agent would always be available. Furthermore, evidence was obtained that other unidentified H donators present in the cell can also bring about the reduction. Very recently Aubel [1938] has shown that washed suspensions of *Bact. coli* can reduce NO_2 (and therefore NO_3) to NH_3 using glucose as H donator. It is perhaps significant that glucose is fermented by *Bact. coli* with production of H_2. Lactate and succinate were unable to bring about the reduction of NO_2. Aubel also obtained some evidence that hyponitrite and hydroxylamine may be intermediates in the reduction of NO_2 by glucose with *Bact. coli*. The evidence is not very convincing as no data are given; it is simply stated "Il a été en outre possible de déceler des traces notables d'acide hyponitreux et d'hydroxylamine dans l'expérience *b*, en appliquant l'excellente technique de Lemoigne, Monguillon et Desvaux." It is not stated if HNO and NH_2OH can be reduced to NH_3 by this strain of *Bact. coli* in the presence of glucose.

It would seem that the reduction of NO_3 to NH_3 by bacteria is more general than supposed, and that the reduction must be seriously considered in assessing the importance of the oxidation of NH_3 to NO_3 by other micro-organisms in the general circulation of N in nature.

Summary

1. Washed suspensions of *Cl. welchii* are able to catalyse the reductions of nitrate, nitrite and hydroxylamine to NH_3 by molecular H_2.

2. During the reduction of nitrate an appearance and final disappearance of nitrite can be demonstrated. This and other evidence makes it clear that nitrite is an intermediate in the reduction of nitrate.

3. There is some evidence (though not conclusive) that hydroxylamine may be an intermediate in the further reduction of nitrite.

4. Two strains of *Bact. coli* tested also brought about the reductions of nitrate, nitrite and hydroxylamine to NH_3 in the presence of molecular H_2.

I wish to express my deep gratitude to Miss M. Robertson for directions as to the maintenance and growth of *Cl. welchii* cultures. I wish also to thank Dr P. Fildes for suggestions and Sir F. G. Hopkins and Dr M. Stephenson for their interest and advice.

REFERENCES

Aubel (1938). *C.R. Soc. Biol., Paris*, **128**, 45.
Aubel, Schwarzkopf & Glaser (1937). *C.R. Soc. Biol., Paris*, **126**, 1142.
Clifton, Mueller & Rogers (1935). *J. Immunol.* **29**, 377.
Conway & Byrne (1933). *Biochem. J.* **27**, 419.
Endres & Kaufman (1937). *Liebigs Ann.* **530**, 184.
Green, Stickland & Tarr (1934). *Biochem. J.* **28**, 1812.
Quastel & Stephenson (1925). *Biochem. J.* **19**, 660.
—— —— & Whetham (1925). *Biochem. J.* **19**, 304.
Stephenson & Stickland (1931). *Biochem. J.* **25**, 205.
Stickland (1931). *Biochem. J.* **25**, 1543.
Stocklasa (1908). *Zbl. Bakt.* II, **21**, 620, 879.
—— & Vítek (1905). *Zbl. Bakt.* II, **14**, 102.

CCLX. OBSERVATIONS ON THE STABILITY OF XANTHINE OXIDASE

By FLORA JANE PHILPOT

From the Department of Biochemistry, Oxford

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In the course of work on the isolation of xanthine oxidase from milk, much inconvenience arose from the instability of the preparations, which increased with each stage of purification. Rapid inactivation occurred when the bulk of the casein had been removed by precipitation with Na_2SO_4 and the diluted supernatant liquid was incubated at 38° for purposes of testing. The inactivation could be prevented by restoring the casein. This led to the hope that a non-protein stabilizer might be found, and, as described below, this hope has been fulfilled.

Experimental

All tests were carried out by the Thunberg technique using the following quantities: 0·1 *M* phosphate buffer, *p*H 7·0, 2·5 ml.; 1 in 5000 methylene blue, 0·5 ml.; 1·0 *M* glycine, 0·5 ml.; 0·0132 *M* xanthine, 0·2 ml.; the final volume was made up to 5·0 ml. Unless otherwise mentioned, the xanthine was added from the hollow stopper after temperature equilibration at 38°.

Results

Table I shows that two-fold dilution of a whey preparation greatly affects its stability. Table II shows the restoration of stability by glycine. Table III shows that reactivation by glycine is also dependent on the time of incubation with glycine.

Table I. *The effect of dilution*

Incubation time in min.	Reduction time: 0·5 ml. whey (min. sec.)	Reduction time: 0·25 ml. whey (min. sec.)
1	5 45	11 40
2	6 05	11 10
3	6 00	15 00
4	5 55	19 00
5	6 05	20 05
10	5 58	23 00
20	6 20	35 00

Table II. *Stabilization by glycine*

1·0 *M* glycine ml.	Enzyme ml.	Incubation time in min.	Reduction time (min. sec.)
—	0·25	2	7 0
—	0·25	10	120 0
0·5	0·25	10	5 30
0·5	0·25	60	7 55
1·0	0·25	60	6 50

The glycine was added to the tubes before incubation.

Table III. *Reactivation by glcyine*

Incubation time (min.) enzyme	Incubation time (min.) enzyme + glycine	Reduction time min. sec.
0	2	5 30
10	0	16 00
10	10	10 15
10	20	7 50

The enzyme was first incubated anaerobically with buffer and methylene blue, then glycine was added and the tubes re-evacuated and incubated, and finally the xanthine was added from the stoppers.

Table IV. *Protection by glycine against copper*

1·0 *M* glycine ml.	Enzyme ml.	0·500125 *M* $CuSO_4$ ml.	Incubation time (min.)	Reduction time min. sec.
—	0·25	—	2	11 10
0·5	0·25	—	2	5 30
—	0·25	0·05	2	150 0
0·5	0·25	0·05	2	5 45
0·5	0·25	0·05	23	5 30

The $CuSO_4$ was added to the other reagents in the tube before incubation.

As the distilled water then being used came from a copper still, it was suspected that the inactivation was due to copper poisoning [Wieland & Mitchell, 1931; Andersson, 1936], and that glycine was acting by forming a copper complex (Table IV). It was shown that glycine protected against the inactivation caused by added copper. Thenceforward all activity tests were done in presence of 0·1 *M* glycine.

In later work it was found that on dialysis at 0°, against large volumes of dilute buffer solutions, there was a loss of activity which could only be partially prevented by adding glycine (0·1 *M*) to the outer liquid and testing in presence of

Table V. *Activation and protection by KCN*

Batch of milk	Preparation	ml.	KCN (final conc.) *M*	Reduction time min. sec.
46	Fat-free milk	1·0	—	2 25
			0·001	1 50
			0·03	1 30
			0·075	2 30
46	Decalcified milk	1·0	—	6 00
			0·015	2 45
			0·03	1 55
			0·045	1 55
			0·075	No reduction
46	Decalcified milk 0·000125 *M* $CuSO_4$	1·0	—	No reduction
			0·015	1 45
			0·03	2 05
			0·045	4 00
48	Fat-free milk	1·0	—	1 45
			0·015	0 45
			0·03	0 40
			0·045	0 48
49	$(NH_4)_2SO_4$ fraction	1·0	—	9 00
			0·0075	4 00
			0·015	3 15

Incubation time 2 min. in all cases.

a high concentration of glycine (1·0 *M*). However, on testing a dialysed preparation in presence of KCN, which has a greater affinity for copper than glycine, it was found that the activity could be not only restored, but actually increased above the original level. A number of different preparations were tested and all, including whole and fat-free milk, were found to be activated, the optimum KCN concentration lying between 0·01 and 0·03 *M*. At lower concentrations the activation is submaximal and at higher concentrations destruction of the enzyme occurs, as found by Dixon & Thurlow [1925]. KCN was also found to protect against inactivation by added copper. The KCN and xanthine were added together to the other reagents from the stopper in order to avoid the destruction produced by KCN alone [Dixon & Keilin, 1936]. As with glycine, the reactivation by KCN, after incubation at 38°, appears to be dependent on time. If tests are carried out in absence of glycine it is found that the reduction time depends on the length of the preliminary incubation. The interpretation of these results is, however, complicated by the fact that the enzyme cannot be incubated alone with KCN without being destroyed.

Discussion

Wieland & Mitchell [1931] stated that KCN does not protect the enzyme against copper poisoning, but they did not test concentrations higher than 0·001 *M*. On the other hand, the high concentration required here suggests that the poison concerned is not silver, gold or mercury (cf. Wieland & Mitchell).

It appears from this that, during the process of purification, xanthine oxidase easily becomes inactivated by traces of copper, but it is not certain where the copper comes from. In the earlier stages of the work the distilled water contained traces, but since then a change has been made to a pyrex glass still. Boyden & Potter [1937] mention that their cellophane dialysis sacks contained copper. The cellophane used here does not contain enough to inactivate more than one batch of 50 ml. of enzyme preparation, and the same sack was used several times over, dialysis producing the same inactivation each time. Since there are traces of copper in milk [Davies, 1936], it seems more probable that the inactivation on dialysis is due to the removal of some substance which normally protects the enzyme against copper poisoning.

The inactivation of the enzyme does not appear to be due to the disappearance of —SH groups, for the following reasons.

(1) Milk has no detectable —SH groups.

(2) The concentration of KCN which activates milk instantaneously has only a very slow reducing action on the —SS— groups in milk.

(3) Addition of cysteine, when testing the activity of milk, causes no activation.

From the practical point of view, if accurate activity measurements are required, it is recommended to use both glycine (final conc. 0·1 *M*) and KCN (final conc. 0·01–0·03 *M*). Since KCN can only be added together with the xanthine, after temperature equilibration is complete, it is advisable to retain glycine to avoid the inactivation which may occur during that time.

Summary

1. Glycine and cyanide can increase the activity of xanthine oxidase in whole milk and concentrates.

2. This is probably due to removal of copper inhibition.

3. The loss of activity of some preparations on dialysis or incubation can be wholly or partly reversed in the above way.

I am grateful to Prof. R. A. Peters for his interest in this work, to Mr J. St L. Philpot for advice, and to Mr R. Gray for help with experimental work in the early stages.

REFERENCES

Andersson (1936). *Hoppe-seyl. Z.* **242**, 205.
Boyden & Potter (1937). *J. biol. Chem.* **122**, 285.
Davies (1936). The chemistry of milk. Chapman, Hall & Co., London.
Dixon & Thurlow (1925). *Biochem. J.* **18**, 976.
—— & Keilin (1936). *Proc. roy. Soc.* B, **119**, 159.
Wieland & Mitchell (1931). *Liebigs Ann.* **492**, 156

CCLXI. INVESTIGATIONS INTO THE METHOD OF ESTIMATING VITAMIN E

III. THE RELATION BETWEEN DOSAGE AND RESPONSE TO VITAMIN E

By ALFRED LOUIS BACHARACH

From the Biochemical Department, Glaxo Laboratories, Greenford

(*Received 27 September 1938*)

In previous communications [Bacharach *et al.* 1937; Bacharach & Allchorne, 1938] we proposed the use of virgin rats as test animals for vitamin E. We also [Bacharach & Allchorne, 1938] defined the sense in which we use the terms "positive response", "live litter" and "fertility rate" or "percentage fertility".

It must be emphasized that the biological assay of any vitamin cannot be carried out until

(1) A stable preparation of the vitamin is available in adequate amounts for use as a standard.

(2) A unit has been defined in terms of this standard.

(3) Information is available as to the relation between response to the vitamin and dosage.

For vitamin E, condition 1, and therefore condition 2, is not at present fulfilled. The need for an agreed standard and a defined unit may of course be circumvented if the pure vitamin, such as one of the tocopherols, becomes available instead. Assays could then be expressed as vitamin E activity in terms of an equivalent weight of tocopherol, but this also could seldom be done with even approximate accuracy unless condition 3 were satisfied.

Having these considerations in mind, we turned our attention about two years ago to establishing the relation between response to vitamin E and dosage, with a view to constructing an average response curve. Such a curve would prove of use for assay purposes when conditions 1 and 2 are satisfied, but it could meanwhile be of service in permitting relative vitamin E activities to be assigned to two materials.

In any endeavour to construct a response curve involving the use of large numbers of animals, a vicious circle is likely to be established. The test must be extended over a considerable period of time, but, in the absence of a quantitative method of test and an agreed standard, there is no convincing means of demonstrating that the test substance has itself undergone no change during the test period.

Precise significance must be given to the word "dosage" in all vitamin tests. For reasons that will be mentioned below we make use of the total dose administered to each animal, that is, the product of the daily dose and the number of days of administration, when the dose is uniformly divided over that number of days.

The practical importance of some of the considerations discussed above was brought forcibly home to us during our first attempt to establish a dosage-response curve, and again later. A large sample of freshly extracted wheat-germ oil was placed in a stoppered bottle and kept in the refrigerator. Vitamin E-deficient female rats were given doses of this oil at several levels, the numbers

of animals that had been submitted to each dose by any given date being kept approximately equal. Examination of the results after many weeks' experiment pointed to a falling activity of the oil, or to an altered sensitivity of the animals, or both (Table I). The former seemed especially probable as the oil was found after 9 months of use to have developed a peroxide value of 43; this had risen to 58 some 10 weeks later. It seemed to us almost certain that the oil must also have undergone loss of vitamin E during the period of test. This made it impossible to do as originally planned, namely, to compare the vitamin E thresholds of virgin and of R.P. animals, a matter that is also referred to in our earlier publication [Bacharach & Allchorne, 1938]. Examination of our figures showed irregular fluctuations in the responses both of R.P. and of virgin animals, and the experiment was abandoned. A similar attempt to construct a curve for response of R.P. animals to cotton-seed oil was abandoned for similar reasons. A dose giving 33 % fertility with R.P. animals gave 100 % and 38 % with 11 and 8 virgin animals respectively. At other levels virgin animals showed responses roughly graded to dosage, thus

Dose (mg.)	No. of animals	Fertility %
562·5	25	4
1125	16	19
1687·5	19	74

This experiment was carried on for about 6 months. Comparison of the above figures with those shown for virgin animals in Table I illustrates the difficulties of evaluating quantitatively one substance in terms of the other.

Table I. *Effect of wheat-germ oil on fertility rates*

(No. of animals in brackets)

		R.P. animals			
			Groups in rough chronological order		
Dose mg. (total)	Virgin animals	Total	1st	2nd	3rd
112·5	4·5 (22)	0 (16)	0 (10)	0 (6)	—
562·5	0 (8)	10 (21)	20 (10)	0 (10)	0 (1)
1125·0	17 (12)	25 (33)	50 (10)	10 (10)	10 (10)
1687·5	57 (7)	68 (31)	80 (10)	90 (10)	40 (10)

We decided next to avoid some of the pitfalls inherent in the use of vegetable oils subject to peroxidation, by making use of a concentrate whose satisfactory biological activity had first been verified in a short exploratory test. This sample, from which most of the sterols had been removed by simple freezing, consisted of unsaponifiable matter from freshly prepared wheat-germ oil; it was sealed off under N_2 in quantities of between 3 and 5 g. and kept in the refrigerator. Fresh tubes were opened as they were wanted and the contents of only one tube were in use at any one time. Further, attempts were again made to keep the numbers of animals at the various levels of dose as nearly equal as possible up to any given time, in order that any slight fall in vitamin E dosage might affect animals on the different doses to roughly the same extent.

The results of this work are shown in Table II and in Fig. 1, which we believe to be the first published curve showing the relation between dosage and response to vitamin E. In this curve the dose has been expressed in arbitrary units; actually, one "unit" corresponded with a total dose of 5·625 mg. of the wheat-germ oil concentrate.

Table II. *Relation between total dosage of wheat-germ oil concentrate and fertility rate*

Dose mg. (total)	Animals		Fertility rate (%)
	Implanted	Fertile	
—	460	0	0
5·625	46	7	15·2
11·25	64	20	31·3
22·50	40	35	87·5
45·00	31	29	93·6
90·00	31	30	96·8
180·00	20	20	100

Some of the animals in this test received their total dose divided uniformly over a period of 5 days and others over a period of 10 days. During the earlier part of this test it appeared to us that the longer dosage period was proving more effective than the shorter. However, as time went on and the increasing numbers of animals at each level gave to the respective fertility rates an increasing significance, we were unable clearly to establish this difference; for the purpose of constructing the curve and Table both periods of dosage have been included, as well as a period of 8 days for some of the animals receiving 90 mg. of extract (16 "units").

We still consider it possible that the method of distributing doses with time may have some effect on response, but it seems clear that only the use of a much larger number of animals could demonstrate a significant difference between, for example, the division of the total dose over 5 days or over 10 days. It is certainly desirable therefore, in any comparative tests, to make use of a fixed dosage period; we ourselves have standardized this at 10 days. The effect of dosage period on response has, of course, been recorded for other vitamins [cf. Bacharach *et al.* 1936].

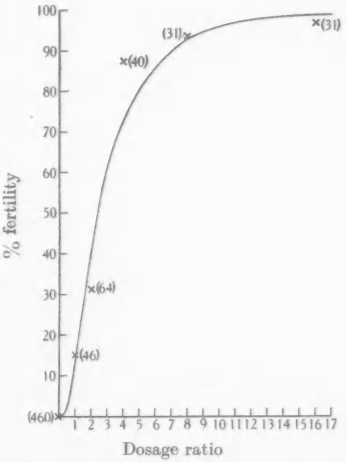

Fig. 1. Dosage-response curve for vitamin E. This curve is constructed from the equation to the regression line, calculated as described. × = fertility found by experiment. Figures in brackets denote nos. of animals. 20 animals receiving a dose with ratio 32 gave 100% fertility, the calculated value being 99·9%. This value was not used in calculating the equation to the regression line, and is not shown in the figure.

The curve shows certain features that call for comment. Theoretically, since it illustrates a typical instance of so-called "quantal" response, it should be sigmoid. It is due to the relatively small number of animals used that this sigmoid structure is not revealed by the lower doses and fertility rates. If, for example, a dose of 1 mg. or 2 mg. produces an extremely low response, about 1 or 2%, this would be expressed in an almost horizontal part of the curve near the origin, but the dose would have to be administered to 100 animals or more for such values to be established. When the curve relating % fertility to dosage ratio is constructed by appropriate transformation of the equation to the regression line, discussed below, the sigmoid portion near the origin becomes evident (see Fig. 1).

Assuming therefore that the relation between dose and fertility is similar to that found for other types of quantal response, the relation between the logarithm of the dose and the normal equivalent deviation of the response, or the "probit" of Bliss [cf. Irwin, 1937], should be linear. A regression line can be calculated from these values by the usual method of least squares. Such a regression line, or the equation to it, can be used not only for comparing the vitamin E contents of two substances, but also for calculating the error of the comparison.

With the co-operation of Dr J. O. Irwin, the figures given in Table II have been used for calculating the equation to the regression line, based on responses to the doses 1, 2, 4, 8 and 16, and it is found to be

$$y = 0{\cdot}87x + 3{\cdot}87,$$

where y is the probit of the response and x is the logarithm (to base 2) of the ratio of the dose to 5·625 mg. By further refinement it is possible to take into account the group of animals that showed a 100 % response to the dose ratio of 32. The derived equation is for a line with a slightly steeper slope. The difference between the results obtained from the corrected and the uncorrected equations lies so far within the range of experimental error inherent in this type of test that the extra labour involved in calculating the corrected equation is not justified.

From the equation the particular concentrate used is found to have a mean fertility dose of 2·5 "units" (13·8 mg.), with probable limits of error, for P = 0·99, 71 % to 141 %, and, for P = 0·95, 77 % to 129 %. Further, it has been calculated that the relation between probits and logarithms of doses shows no significant departure from linearity.

The equation to the regression line makes it possible, albeit with an accuracy inversely related to the square root of the number of animals used, to find with a single group the mean fertility dose of a source of vitamin E; the more the found fertility departs from a fertility rate of 50 %, the greater the error of the test. By a simple transformation of the equation to the regression line, it can be shown that

$$\log D_M = \log D_T - 0{\cdot}35y_T + 1{\cdot}74,$$

where D_M and D_T are the mean fertility dose and the test dose respectively, and y_T is the probit of the response given to the dose D_T. In the example illustrated in Table IV and mentioned again below, the two values for the mean fertility dose obtained from the groups of animals given the two doses of wheat-germ oil are calculated to be 300 and 210 mg., with a weighted mean of 260 mg. The weighted mean value obtained by graphical methods from the standard response curve was 280 mg. The direct dosage-response curve is very steep, and for most purposes it will be found sufficient to use it rather than the derived regression line, as the above example shows, provided that the responses involved fall on the approximately straight part of the curve.

Certain practical considerations emerge from the exceptional steepness of the curve. A 300 % increase of dose, from 1 to 4 "units", has brought about a nearly 500 % increase in the fertility rate, from 13 % to over 70 % fertility. When larger numbers of animals are taken into account, experimentally determined points should lie still closer to the plotted curve, but it seems unlikely that its general shape would be materially changed, and one must therefore anticipate that testing vitamin E by the fertility rate method at levels producing below 25 % or above 75 % fertility is liable to show a high degree of error. On the other hand, the steepness of the curve has a compensating advantage;

provided that a fertility rate near to 50 % results from the administration of a particular dose, the equality of that dose with a dose of other material giving the same fertility rate can be asserted with some confidence. The steepness of the curve, in other words, means that the range over which tests can be accurately carried out is restricted, but that within this range, the accuracy is considerably higher than might have been anticipated for results obtained with small numbers of animals.

A response curve of the type shown affords certain useful information. For example, it may be required to know how much vitamin E is to be added to some synthetic diet in order to make it an adequate source for full fertility. Inspection of the curve shows that twice the mean fertility dose is insufficient, that three times that dose is nearly adequate, and that six times is almost certainly a safe addition for the purpose.

Since, however, the curve is based on the behaviour of completely deficient animals, it is clear that much smaller amounts would be required daily if they were administered throughout the lives of the animals. If M be the mean fertility dose found for a substance administered over 10 days to virgin animals about 100 days old, presumably a daily dose of $6M/100$ would be quite adequate if given daily from birth, or even from weaning. A daily dose of $M/10$ would allow a margin for any inefficient storage of the vitamin during infancy and adolescence. A sample of wheat-germ oil whose M.F.D. is found to be 1 g. should confer full fertility if fed from weaning in daily doses of 100 mg. to animals on a diet otherwise completely free from vitamin E, always assuming that the oil undergoes no serious deterioration during its period of administration. We have records of 87 % fertility (15 animals) and 84 % fertility (6 animals) for two different samples of wheat-germ oil given in daily prophylactic doses of 112 mg.

Table III. *Vitamin E activities of α- and β-tocopherol*

Substance	Dose mg.	No. of animals	Fertility rate (%)
α-Tocopherol	1·125	5	40
	2·25	6	50
	4·50	5	100
β-Tocopherol	0·72	6	0
	1·8	1	0
	2·25	9	0
	2·70	1	100
	3·6	6	83

It may be of interest to give a few examples of the use of the response curve in comparative tests. We have published elsewhere [Bacharach, 1938, 2] figures for a test carried out with highly purified samples of the allophanates of α- and β-tocopherol. Material was available for administration to a few more animals after we had made that communication, and the consequently revised comparison is shown in Table III. The M.F.D. of α-tocopherol is considered from the results shown to lie in the neighbourhood of 1·5 mg. (0·8 × 1·9 mg.; the factor 0·8 represents conversion of the allophanate into the free alcohol). The activity of β-tocopherol appears to be rather over half that of α-tocopherol, but the error of this test is extremely high. Indeed, it is doubtful whether these or any other published figures finally establish a difference in biological activity of the two forms of tocopherol. Their activities call for some further comment. Concentrates used for isolation of the crystalline allophanates generally exhibit a M.F.D. of something between 15 and 30 mg. At worst, then, they should

contain 5% of α-tocopherol or rather under 10% of the β form; at best they should contain twice these quantities. Yet yields of even the crude mixed allophanates have never been reported as much above 1% of such concentrates, suggesting that the process of isolation may result in loss or destruction of up to 90% of the active substances [cf. Bacharach, 1938, 1].

Table IV. *Vitamin E activities of wheat-germ oils and concentrates*

	Dose mg.	Fertility rate (%)	M.F.D. from curve (mg.)	No. of animals	Weighted M.F.D. (mg.)
Wheat-germ oil A	255	43	270	7	280
	450	83	310	6	
Wheat-germ oil B	450	0	>1100	7	700
	900	84	600	6	
Wheat-germ oil C	450	75	320	4	250
	675	100	<250	6	
Concentrate from C	20	57	18	7	15
	40	100	<15	7	

A comparison of two samples of wheat-germ oil is given in Table IV. From the figures it is concluded that one oil is about three times richer than the other in vitamin E. Here again the error is very large; it is quite possible that the activities of the oils do not differ by more than 50%. Again, a sample of wheat-germ oil concentrate was compared with the oil from which it was made. It seems that an approximately 16-fold concentration has been achieved.

Whether or not the relation between dosage and response found under our conditions of test would also apply generally is by no means to be taken for granted. Nevertheless, we believe our procedure to be of general application and to show clearly that comparisons of vitamin E activity should always be made by establishing, under as nearly identical conditions as possible, the mean fertility doses of the materials in question.

I would like to express my indebtedness to Miss E. Allchorne, who had charge of all the animal experiments discussed in this paper, and to Miss E. van Rossum who assisted her. I wish also to thank Dr J. O. Irwin most cordially for his kind advice on the statistical aspects of this paper.

REFERENCES

Bacharach (1938, 1). *Nutrit. Abs. Rev.* **7**, 815.
—— (1938, 2). *Nature, Lond.*, **142**, 35.
—— & Allchorne (1938). *Biochem. J.* **32**, 1298.
—— —— & Glynn (1936). *Biochem. J.* **30**, 2004.
—— —— —— (1937). *Biochem. J.* **31**, 2287.
Irwin (1937). *Suppl. J. Roy. Stat. Soc.* **4**, 1.

ADDENDUM

We have had the privilege of reading in proof the recent paper by Mason & Bryan (*Biochem. J.* 1938, **32**, 1785), wherein they point out that we have omitted in our earlier publications to state whether or no our experimental animals (that is, the young does ultimately to be used in the test) themselves

had access to their mothers' diet before they were weaned and, if so, what that diet consisted of. We would seize this opportunity to rectify the omission. No steps are taken to prevent the sucklings from eating their mothers' diet, which is replaced by our standard vitamin E-free diet either the day after weaning or, if on that day they weigh under 35 g., when they have reached this weight, which is at most 2 days later. The stock diet that these young does may therefore be wholly or partly consuming for 10 to 15 days, preceding their transference to the vitamin E-free diet, is practically identical with that described in an earlier publication (Bacharach, *Biochem. J.* 1933, **27**, 5). It consists of wholemeal flour, full-cream dried milk and yeast extract, supplemented with limited amounts of green leaves, liver (or lean meat) and fish: the supplements, however, are withdrawn towards the end of their mother's pregnancy and are not restored until after the litter has been weaned.

CCLXII. THE EFFECT OF DIFFERENT METHODS OF DRYING ON THE BIOLOGICAL VALUE AND DIGESTIBILITY OF THE PROTEINS AND ON THE CAROTENE CONTENT OF GRASS

By STEPHEN BARTLETT, KATHLEEN MARY HENRY, STANISŁAW KAZIMIERZ KON, LESLIE WILLIAM OSBORNE, SYDNEY YAN THOMPSON and JOSEPH TINSLEY

From the National Institute for Research in Dairying, University of Reading

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One of the outstanding features claimed for modern methods of artificial grass drying is that they involve but little destruction of the nutritive properties of the grass. The high temperatures attained during the process, however, raise the question of heat injury to the proteins. It is well known that heating lowers the biological value of the nitrogen of various foodstuffs [Morgan, 1931; Kon & Markuze, 1931; Fixsen & Jackson, 1932; Maynard & Tunison, 1932; Morgan & Kern, 1934; Morris *et al.* 1936]. Morris *et al.* [1936] found, on the whole, little difference between the biological values for cows of the proteins of fresh and artificially dried grass, though with spring grass there was an indication that the dried product was inferior. In view of the increasing use of artificially dried grass in the feeding of pigs and of poultry and of marked differences between herbivora and omnivora in the utilization of the nitrogen of feeds it was of interest to study on another animal the effect of heat treatment on the proteins of grass. Rats have been extensively used for such tests. They respond readily to even small alterations in the chemical make-up of the nitrogenous constituents of their diets. Further, it is probable that they are less able than ruminants to utilize breakdown products of proteins and are therefore more sensitive in indicating the possible detrimental effects of heating. It is probably safe to assume that if a rat test discloses no difference between two products, a feeding test on cows would likewise show no difference. On the other hand, a definite difference in this respect found with rats would certainly call for a further investigation with ruminants.

For these reasons it was decided to carry out nitrogen balance experiments on rats. The carotene content of the grasses dried under various conditions was also studied, as well as the rate of destruction of carotene in the process of natural curing.

Experimental

(1) *The drying and preparation of grass*

Compared with that of herbivora, the capacity of the intestinal tract of the rat is limited and it was necessary in these experiments to cut down as far as possible the bulk of the diet by selecting a grass of very high protein content. For the same reason (apart from other difficulties) it was not possible to feed the grass fresh and only dry samples could be compared.

Grass was taken from permanent grassland with a first class sward about 4–6 in. in length. The chief plants in the herbage were wild white clover and perennial rye grass. The mower was run over the meadow on 25 June 1937 to remove all long grass and on 21 July the grass was cut with a close cutting mower with an elevator attached so that the freshly cut grass was loaded on a cart immediately and taken to the farm buildings for drying.

The details of the various treatments applied to this batch of grass were as follows.

(*a*) Grass dried artificially in a grass drier at the normal running temperature (300° F.).

The drier used was a Kaloroil drier of the rotating drum type heated by oil fuel.[1] A full description of this type of drier will be found in the report by Roberts [1937]. The inlet gases were heated to about 300° F. (variation 275–325° F.) but the outlet temperature could not be recorded.

The grass was removed from the drier as soon as it was sufficiently dry (15–30 min.) and it is probable that only the outside of the grass ever reached a temperature approaching 300° F. The freshly dried grass was crisp but after removal from the drier quickly became less powdery. The interval between cutting and drying this sample was 6 hr.—i.e. 2 hr. on the cart and 4 hr. exposure on a wooden platform under a Dutch barn. The condition of this sample after drying was excellent and it was dark green in colour. It was stored in sacks for 15 days and was then made into bales under high pressure and was covered with sacking.

(*b*) Grass dried artificially in a grass drier at low temperature (170° F.).

The interval between cutting and drying this sample was 6½ hr. It was dried in the same plant as the first sample but the temperature of the inlet gases was kept at about 170° F. (variation 150–200° F.). The time required for drying was much longer and it was necessary to keep the grass in the drum for about an hour (50–70 min.). A well-dried dark green sample of grass was eventually obtained and the after treatment was the same as for the first sample.

(*c*) Grass dried artificially in a current of hot air.

A third batch of the grass was placed half an hour after cutting in a tunnel-shaped drying oven through which passed a current of air heated by a hot water radiator and propelled by a fan. The temperature of the inlet air was about 135° F. (variation 104–142° F.). The time of drying was 22 hr. This sample was sacked but not pressure-baled.

(*d*) Grass sun-dried under natural conditions (hay).

A further batch of grass was unloaded from the cart 2 hr. after cutting. It was intended to place the grass in the open at once but as heavy rain fell while unloading it was left overnight indoors spread on a floor. The depth of the layer was about 8 in. and it was turned to avoid undue fermentation. Twenty-one hr. after cutting the grass was spread in the open in a layer about 3 in. thick. The weather to which the grass was subjected was unsettled and dull, and heavy rain fell in the 36th hr. after cutting. The grass was turned on the 3rd day. In order to complete the drying on the 5th day after cutting the grass was turned three times and finally placed in a small stack and thatched with straw. The hay had lost most of its green colour and parts were slightly blackened but would be described by a farmer as moderate quality hay. Ten days later this hay was pressure-baled and the bales were covered with sacking for storage.

In addition to these samples, a further batch of grass was cut from the same meadow one day later and was treated as follows.

[1] We are indebted to Messrs Kaloroil Burners, Ltd., for the loan of the drier.

(*e*) Grass dried in air at normal temperature without sun or rain.

The grass was cut, carted and off-loaded after an hour. It was spread about 3 in. deep on a concrete floor in a room with screened windows. The grass was turned twice daily to prevent fermentation and was thoroughly air-dried in 12 days. It was pressure-baled 14 days after cutting and the bales were covered up with sacking. The hay lost much of its green colour during curing but otherwise appeared to be of very good quality.

Finally, to study the rate of destruction of carotene in the course of curing, part of a different meadow was scythed between 11 and 11.30 a.m. at the beginning of September, the grass was left in the field in a layer of about 3 in. and samples were withdrawn at intervals for carotene estimations. This field had been previously cut at the beginning of June. Between then and the second cutting the weather was, on the whole, very dry, and the grass made little growth; it was 6–7 in. high when cut and rather parched. During curing the weather was, on the whole, bright and sunny with no rain.

(2) *Determination of the biological value and digestibility of the proteins of dried grass*

It was technically not possible to compare simultaneously more than three types of dried grass and the tests were therefore limited to three products of practical importance: two samples of grass dried in the drier at high and low temperature and the sample of hay. The feeding experiments started $5\frac{1}{2}$ months after the grass had been dried.

The grasses dried at high and low temperature contained respectively 23·5 % and 23·1 % of protein (N × 6·25) on the dry basis. The sun-cured sample contained more, 25·1 %, probably owing to relatively greater loss of fermentable non-nitrogenous constituents.

The grasses were milled to a fine powder and were mixed in suitable proportions with a "nitrogen-free" basal diet to yield mixtures containing about 9 % protein (N × 6·25) on the dry basis. The "nitrogen-free" diet had the following composition:

Butter fat	17
Rice starch	51
Potato starch	10
Sugar	17
Salts (Steenbock 40)	5

The biological value and true digestibility of the grasses were measured by the method of Mitchell [Mitchell, 1924; Mitchell & Carman, 1926] as described in detail by Henry *et al.* [1937]. Because of the nature of the grass diets, it was necessary to mix them for feeding with a relatively large quantity of water (more than $1\frac{1}{2}$ times the weight of the diet). Twelve rats were used and on each the three types of grass were tested in turn. The results are given in Tables I, II and III. Table I gives the individual nitrogen metabolism data, Table II gives average values and Table III contains a statistical examination of differences in biological values. Figures for true digestibilities were so similar (Table II) that statistical tests were considered superfluous. The statistical method used was that of the paired *t* test of "Student" [1908; 1925] as described by White [1937]. When comparing two substances results obtained on the same rat in different periods were paired. The results show quite conclusively that sun-drying was more detrimental than artificial drying at either high or low temperature. The figures also indicate that the proteins of grass dried at low temperature were inferior to those of the high-temperature grass but the evidence to this

Table I. *Nitrogen metabolism; individual data for 6-day periods*

Rat no.	Type of grass*	Initial wt. g.	Final wt. g.	Diet intake† g.	N intake mg.	Faecal N mg.	N in urine mg.	Biological value	True digestibility
				Low egg-nitrogen diet					
1	—	64	70	46·11	—	101·5	100·0	—	—
2	—	65	69	46·94	—	89·8	102·2	—	—
3	—	69	73	44·91	—	92·9	110·9	—	—
4	—	59	64	37·67	—	93·6	98·2	—	—
5	—	58	60	31·82	—	73·5	111·1	—	—
6	—	60	68	49·59	—	105·0	113·8	—	—
7	—	64	71	43·73	—	103·8	106·8	—	—
8	—	62	66	37·38	—	75·6	101·2	—	—
9	—	64	73	47·45	—	97·8	106·2	—	—
10	—	60	63	40·61	—	99·4	108·3	—	—
11	—	59	62	35·14	—	90·4	98·4	—	—
12	—	61	62	38·86	—	98·0	106·3	—	—
				1st grass period					
1	S	68	69	40·31	566·4	265·7	297·5	51·7	69·9
2	H	71	72	38·59	544·9	275·1	257·9	57·3	64·5
3	L	72	75	44·54	624·5	311·8	274·6	59·7	64·9
4	S	61	59	34·93	490·8	257·9	282·2	39·5	64·3
5	H	64	66	40·32	569·3	302·6	241·1	66·0	63·9
6	L	64	64	35·54	498·3	261·6	264·9	50·2	63·1
7	S	71	67	36·70	515·6	264·8	269·8	50·8	65·7
8	H	70	72	42·78	604·1	308·6	273·8	57·2	63·5
9	L	71	73	41·17	577·2	282·8	288·3	53·1	66·5
10	S	63	63	42·04	590·7	297·0	328·1	43·5	67·1
11	H	62	68	47·51	670·8	331·0	261·5	65·9	69·2
12	L	62	66	44·62	625·6	312·6	294·6	55·8	68·5
				2nd grass period					
1	H	77	79	45·39	640·9	373·2	225·7	73·4	59·6
2	L	77	82	48·14	674·9	364·2	255·3	67·4	62·5
3	S	79	80	47·49	667·2	376·1	275·1	59·1	58·5
4	H	64	69	42·34	597·8	299·3	228·0	66·9	65·7
5	L	70	73	45·07	631·9	322·5	246·3	71·0	66·9
6	S	68	70	43·75	614·7	336·9	284·1	54·0	61·3
7	H	72	83	55·70	786·5	381·6	262·1	72·0	68·6
8	L	73	77	46·52	652·2	348·4	272·5	60·3	61·6
9	S	75	74	44·41	623·9	333·6	316·5	47·4	62·8
10	H	68	71	45·58	643·6	261·5	245·8	72·3	76·5
11	L	71	73	45·86	643·0	344·2	239·9	69·7	66·1
12	S	66	68	44·30	622·4	333·7	299·8	52·0	65·3
				3rd grass period					
1	L	78	81	53·34	747·8	411·7	347·4	54·8	64·0
2	S	79	83	51·56	724·4	370·4	319·7	58·1	66·8
3	H	84	94	57·96	818·4	444·6	257·0	73·0	60·6
4	L	71	78	49·42	692·9	339·5	263·3	65·4	66·0
5	S	76	78	49·93	701·5	355·4	314·6	60·4	68·0
6	H	73	83	57·23	808·1	430·7	269·0	70·6	63·2
7	L	85	91	56·00	785·1	423·8	276·6	68·2	63·5
8	S	75	81	53·82	756·2	404·2	331·3	53·7	61·8
9	H	75	85	57·14	806·8	426·5	294·5	65·3	64·1
10	L	76	84	56·31	789·5	382·1	278·8	70·3	68·8
11	S	74	76	50·86	714·6	418·9	309·0	52·1	61·6
12	H	71	79	52·10	735·7	366·5	281·7	66·7	69·5
				Low egg-nitrogen diet					
1	—	80	84	38·57	—	108·9	138·6	—	—
2	—	85	86	34·18	—	92·8	122·0	—	—
3	—	96	101	48·98	—	103·9	130·8	—	—
4	—	83	95	55·09	—	108·6	119·6	—	—
5	—	78	80	34·53	—	94·9	122·6	—	—
6	—	87	96	57·36	—	137·7	132·4	—	—
7	—	95	105	59·13	—	146·6	126·6	—	—
8	—	85	91	41·92	—	91·6	126·4	—	—
9	—	92	93	43·32	—	108·5	130·7	—	—
10	—	87	95	52·81	—	126·6	125·0	—	—
11	—	77	81	40·19	—	118·0	116·2	—	—
12	—	85	85	38·41	—	107·2	120·0	—	—

* S=sun-dried. H=artificially dried at high temperature. L=artificially dried at low temperature.

† On the dry basis.

Table II. *Biological values and true digestibilities of three types of dried grass*

	Sun-dried		Artificially dried: High temperature		Artificially dried: Low temperature	
Period	Biological value	True digestibility	Biological value	True digestibility	Biological value	True digestibility
I	46·38	66·75	61·60	65·28	54·70	65·75
II	53·11	61·97	71·15	67·60	67·10	64·28
III	56·08	64·55	68·90	64·35	64·68	65·57
Mean	51·86	64·42	67·22	65·74	52·16	65·20

Table III. *Statistical significance of the mean differences in the biological values of the three types of dried grass*

Difference	Standard error of mean	P*
High temperature – Sun-dried	$+15{\cdot}36 \pm 2{\cdot}63$	1 : 5000 Significant
Low temperature – Sun-dried	$+10{\cdot}30 \pm 2{\cdot}81$	1 : 263 Significant
High temperature – Low temperature	$+\ 5{\cdot}06 \pm 2{\cdot}85$	1 : 10 Not significant

* P = probability that a mean difference at least as great as the observed mean difference would have arisen by random sampling from a homogeneous population.

effect is inconclusive (Table III). The lowering of the biological value of the proteins in the sun-curing of grass is most probably associated with the fermentative changes taking place during the process. It is sufficiently marked, as judged on rats, to invite an investigation on ruminants. According to Saakian [1935] artificially dried hay was 20 % higher in feeding value than ordinary hay from the same meadows.

Nevens [1921] has measured on rats the biological value and true digestibility of the proteins of alfalfa (lucerne) hay and obtained values of 62 and 58 respectively. The former figure is definitely higher than that obtained in the present work for sun-cured grass, slightly lower than that for grass dried at high temperature and equal to that for the low-temperature grass. The digestibility coefficient of the lucerne hay was lower than that of the artificially dried grasses which amounted to about 65.

(3) *The effect of drying on the carotene content of the various grasses*

The carotene was estimated by the method of Ferguson & Bishop [1936]. The results will be found in Tables IV and V of which the first gives the carotene

Table IV. *The carotene content of grass dried by various methods*

Method of drying	% moisture	Mg. carotene/100 g. grass: Moist	Mg. carotene/100 g. grass: Dry	Carotene content as % of that present in fresh grass
Fresh grass	88·92	10·4 10·2	49·3 48·5 } 48·9	100
Artificially dried high temperature	10·56	39·1	42·4	86·7
Artificially dried low temperature	12·90	32·1	38·2	78·1
Artificially dried in current of hot air	7·89	39·0	43·7	89·4
Sun-dried under natural conditions (hay)	21·38	9·6	12·2	25·0
Dried without sun at air temperature	18·06	12·6	15·4	31·5

Table V. *Rate of destruction of carotene in the sun-curing of grass*

Hours after cutting	Time of exposure to sunshine* hr. min.	% Moisture	Mg. carotene/100 g. grass		Carotene content as % of that present in fresh grass
			Moist	Dry	
Freshly cut	—	65·27	8·7	25·0	100
1	54	59·05	9·0	22·0	88
3	2 54	47·78	11·0	21·0	84
6	5 27	38·59	10·3	16·8	67·2
24	9 57	35·54	9·9	15·4	61·6
48	16 32	35·09	9·1	13·4	53·6
72	24 32	24·78	4·7	6·3	25·2
96	30 45	16·17	4·4	5·3	21·2

* As measured by a Jordan photographic sunshine recorder.

content of the grasses used in the protein experiment and the second shows the changes in the carotene content of grass in the course of its conversion into hay.

In both cases hay cured under natural conditions contained only about one quarter of the carotene originally present in the fresh grass. Artificial drying at normal running temperature preserved nearly 90 % of the carotene. This figure is in good agreement with the earlier findings of Watson *et al.* [1933]. The drying at the lower temperature was slightly more injurious, most probably because of the longer exposure of the material to the hot gases after it had become relatively dry [Watson, 1934] and it is possible that the same reason would account for the slight lowering of the biological value of the proteins described on p. 2028.

The high figure for the carotene content of the sample of grass dried in a hot air tunnel is rather surprising as the drying took some 24 hr. and air was continuously passing over the grass; on the other hand the temperature of the air never rose above 142° F. and that of the grass must have been even lower for, at any rate, part of the time.

The measurements of the rate of disappearance of carotene when grass is made into hay show a fairly steady fall. The loss after 48 hr. is some 46 % of the original value. This compares unfavourably with the figures given by Greenhill [1936], but it must be remembered that his results were obtained in the course of partial field drying of grass as a preliminary to artificial drying and not in haymaking. On the other hand, the figures agree quite well with the data of Dexter & Moore [1937] for lucerne hay cured in swaths.

Summary

1. Grass was dried by artificial means and also made into hay in the normal way and the effects of the various procedures on the carotene content and on the biological value of the proteins of grass were measured.

2. The biological value and true digestibility of the proteins of grass artificially dried at the normal running temperature (300° F.) in a rotating drum type drier were compared on rats by the method of Mitchell with those of grass dried in the same drier at low temperature (170° F.) and those of sun-cured hay. All three samples were made from one bulk of fresh grass.

3. The following figures were found for biological value and true digestibility: artificially dried grass, high temperature, 67 and 66; artificially dried grass, low temperature, 62 and 65; hay, 52 and 64.

The differences in biological values of the proteins between artificially dried samples and hay were statistically highly significant. Other differences were not.

4. Carotene figures are given for various samples of dried and fresh grass and also measurements of the rate of loss of carotene in the course of sun-curing of grass.

Our thanks are due to Prof. H. D. Kay and Mr J. Mackintosh for advice during the course of these experiments; also to Mr J. E. Campion who assisted in operating the grass-drying plant.

Note added 28 October 1938. Smuts & Malan [1938] have obtained very recently for lucerne meal biological values of 60 and 61 which agree very well with the findings of Nevens [1921].

REFERENCES

Dexter & Moore (1937). *Quart. Bull. Mich. agric. Exp. Sta.* **20**, 75.
Ferguson & Bishop (1936). *Analyst*, **61**, 515.
Fixsen & Jackson (1932). *Biochem. J.* **26**, 1923.
Greenhill (1936). *Emp. J. Exp. Agric.* **4**, 145.
Henry, Kon & Watson (1937). Milk and Nutrition, Part I, p. 37. Reading: Nat. Inst. Res. Dairying.
Kon & Markuze (1931). *Biochem. J.* **25**, 1476.
Maynard & Tunison (1932). *Industr. Engng Chem.* **24**, 1168.
Mitchell (1924). *J. biol. Chem.* **58**, 873.
—— & Carman (1926). *J. biol. Chem.* **68**, 183.
Morgan (1931). *J. biol. Chem.* **90**, 771.
—— & Kern (1934). *J. Nutrit.* **7**, 367.
Morris, Wright & Fowler (1936). *J. Dairy Res.* **7**, 97.
Nevens (1921). *J. Dairy Sci.* **4**, 552.
Roberts (1937). *Rep. Ser. agric. Res. Coun., Lond.*, 2.
Saakian (1935). *Probl. Zivot.* No. 8, 55.
Smuts & Malan (1938). *Onderstepoort J. vet. Sci.* **10**, 207.
"Student" (1908). *Biometrika*, **6**, 1.
—— (1925). *Metron*, **5**, 105.
Watson (1934). *J.R. agric. Soc.* **95**, 103.
—— Drummond, Heilbron & Morton (1933). *Emp. J. exp. Agric.* **1**, 68.
White (1937). Milk and Nutrition, Part I, p. 64. Reading: Nat. Inst. Res. Dairying.

CCLXIII. THE CATATORULIN TEST FOR VITAMIN B_1

By RUDOLPH ALBERT PETERS

From the Department of Biochemistry, Oxford

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THE catatorulin test, using avitaminous pigeon's brain, may still occasionally prove useful, because it is fairly sensitive, reacting to less than $0{\cdot}2\gamma$ vitamin B_1, and because it has a low sensitivity to the phosphoric esters [Peters, 1937]. With the newest thiochrome test [Jansen & Westenbrink, 1938] small amounts of vitamin B_1 are evidently determined, but the Schopfer mould test does not distinguish between the different forms of vitamin [Sinclair, 1937], and the new yeast enzyme test does not separate the free vitamin from its monophosphoric ester. Further there are points of general application in the use of a tissue preparation of this nature for an enzyme test in which it is found that fine grinding or extraction of the tissue leads to loss of activity [McGowan & Peters, 1937]. For these reasons the collective experience of some years with this test is here recorded briefly; in particular, it is believed that a satisfactory theoretical basis has been found for the use of pyruvate and that the conditions have now been defined which make for the maximum degree of sensitivity.

The first catatorulin tests for vitamin B_1 using avitaminous pigeon's brain were based upon the increase in O_2 uptake observed in lactate solution as the result of adding the vitamin [Passmore *et al.* 1933]. This was extended to lactate and pyrophosphate [Kinnersley *et al.* 1935], when it was found that this gave larger differences. Finally it became clear that pyruvate rather than lactate was the essential substrate; attention was therefore diverted to pyruvate [Ogston & Peters, 1936], especially in view of the further increase in the O_2 differences which was thus obtained. At the same time these last changes meant giving up an equation which had served well previously with lactate for the 2nd hour of respiration. Recent papers from this laboratory [Peters, 1936; McGowan & Peters, 1937] show clearly the incorrectness of the theoretical basis of this Michaelis equation in which there was postulated a reversible equilibrium between vitamin B_1 and some essential component of the system. Now we know that a quite different series of reactions is involved. In the absence of vitamin B_1, some component (probably protein) of the pyruvate-oxidase system becomes unstable; this is proved by the fact that the effect of vitamin B_1 tends to be that of maintenance [Peters *et al.* 1935; Westenbrink & Polak, 1937]; the control decreases in respiration rate more rapidly than the sample containing vitamin, and also the washed avitaminous brain tissue gives a poor catatorulin effect which is not improved by the addition of the washings. The steadier values obtained for respiration during the second hour are only in part due to the removal of the residual substrates; they are probably associated with the inactivation of the unstable component of the system; the rate of inactivation increases with rising temperature. In lactate the series of changes summarized by the previous equation is complicated:[1] (*a*) lactate is converted into pyruvate; (*b*) vitamin B_1

[1] Another possible explanation of the catatorulin effect might be that added aneurin merely prevents the dissociation of the last traces of pyrophosphoric ester, but this is not consistent with the trifling effect of vitamin upon the normal brain.

reaches its unstable component by diffusion; (*c*) the vitamin may first have to be phosphorylated, a question which is not yet decided [Peters, 1937; Ochoa & Peters, 1938]. By using pyruvate (*a*) is eliminated.

In arriving at a new theoretical treatment for pyruvate as substrate it must be remembered that the Q_{O_2} observed has three components, (1) residual respiration (not due to pyruvate), (2) residual pyruvate respiration (due to residual traces of vitamin B_1) and (3) catatorulin respiration, due to added vitamin. We may call (1)+(2) *R*. If we assume that the added aneurin *a* acts either as a prosthetic group or coenzyme to some protein, *p*, we can write *ap* as the actual enzyme system. At any time the catatorulin O_2 uptake in the absence of complicating factors depends upon *ap*. Since *p* is very unstable at 38° in absence of aneurin and substrate [Peters, 1936], at any time after 30 min. no more *ap* can be formed; after this time therefore

$$Q_{O_2} \text{ observed} = k\,(R+ap).$$

We should expect the difference between the Q_{O_2} with and without added vitamin to remain constant after the 30 min. period, if the complete system is reasonably stable, because the diminution of the residual respiration (1) will be the same for each, and the residual traces of the pyruvate-oxidase system will remain equally stable. The curves shown in Fig. 1 are consistent with this view, which has been previously expressed.

For the period of respiration ½–2 hr. there is a fairly constant change produced by the aneurin in μl./g./hr., though the general level of respiration is falling. Taking recent experiments consecutively, I have calculated in 28 cases the average variation from the mean over the period 1–2 hr. for the separate half-hours, and have obtained the value $\pm 7{\cdot}8\,\mu$l.; this is only a small percentage of the total values involved. For the period 0·5–1·0 hr. the average difference from the same mean is greater, namely $\pm 15{\cdot}2\,\mu$l. Hence there is experimental justification for the idea that the system once formed remains stable, and proof that the values for the period 1–2 hr. are more consistent (σ for individual figures varying from 300–600 μl. for 1–2 hr. is 22 and for 0·5–1·0 hr. 63).

Further support for the idea that the aneurin system once formed is irreversible is to be found in the failure to obtain substantial catatorulin effects with normal brain mentioned above, and the absence of a catatorulin action in solutions in which normal brain has been previously shaken.

Accepting the hypothesis that the rate of pyruvate oxidation is proportional to the quantity *ap*, theoretically this rate should be exactly proportional to the amount of aneurin added, provided that the active surface adsorbs the aneurin practically quantitatively. Hence, up to a certain limit which is set by the amount of the residual aneurin system still in the avitaminous tissue, increases in rate due to equal increments of aneurin should be equal. There should be a straight line relation between the amount of vitamin and the catatorulin O_2 up to a maximum, which should then at once show no further increase. Fig. 2 (Theory) indicates the kind of curves which would be obtained if this view were correct. The typical experimental curves also shown in Fig. 2 are in general agreement with the hypothesis; they indicate that after the addition of 1·0γ there is in all cases an abrupt discontinuity; in many cases there is practically no increase after 0·5γ. This is consistent with slight variations in the residual vitamin left in different brains and it means that in practice the part of the curve from 0·5–1·0γ is dangerous to use for any but the most approximate estimations. Inspection of the experimental curves obtained during the last few years showed

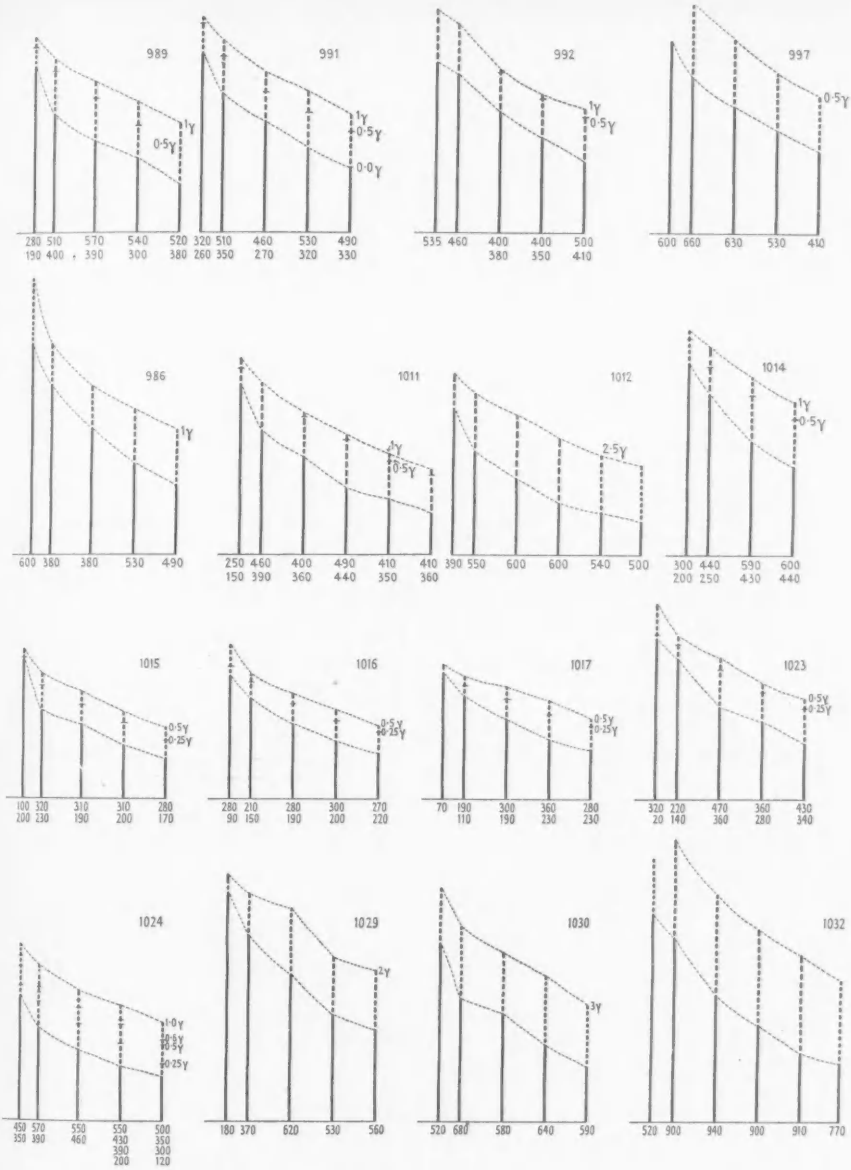

Fig. 1. Catatorulin tests indicating the variation of the respiration rate with time and different amounts of vitamin, indicated as γ on the diagram. Ordinate = rate of respiration, abscissa = time. Each vertical line represents the rate μl./g./hr. of respiration for successive periods, 15, 15, 30, 30, 30 min. The figures below the vertical lines represent the extra O_2 uptake due to vitamin.

several which departed more widely from the hypothesis above than could be attributed to experimental error. This departure is always in the sense that the 0·25γ point is higher than it should be for the straight line relation. Owing to the difficulty of standardizing conditions exactly in this system, it is not worth pursuing this point far; the best values for the catatorulin test will be obtained

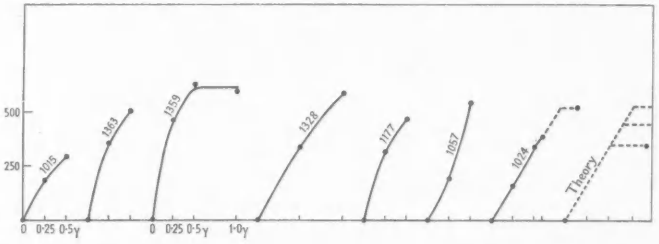

Fig. 2. Curves indicate the change in extra O_2 uptake produced by vitamin in varying concentration. Ordinate = O_2 uptake μl./g./hr.; abscissa = concentration of vitamin; the actual amount is indicated in two experiments; the remainder are drawn to the same scale. Last diagram marked "Theory" is drawn from theoretical considerations.

between 0·25 and 0·5γ, in the region of 0·3γ. I have found, however, that large differences are caused in catatorulin values by not allowing sufficient time for diffusion equilibrium to occur before introducing the bottles into the warm bath; an extreme example of this effect is given in Table I.

Table I. *Effect of allowing time for diffusion on the catatorulin test*

After division of the tissue at the mashing stage, the vitamin was immediately introduced into the bottles. Samples marked "No standing" were quickly filled with O_2 and placed in bath; those "Stood 15 min." were kept at room temperature for this time with shaking.

Extra O_2 uptake due to vitamin for period 1–2 hr., μl./g./hr.

Vitamin added	0·25γ	0·5γ
No standing	556	620
Stood 15 min.	343	523

Here the effect of standing has been to reduce the total O_2 uptake but to improve the divergence between the two concentrations of vitamin. The theoretical straight line is found when the latter values are plotted.

The improvements in method given below are based upon the above principles.

It is not easy to understand why the Michaelis type of equation, proposed by Passmore *et al.* [1933], held good for lactate. Peters *et al.* [1935][1] found that the pyruvate formed from lactate in presence of vitamin immediately underwent further oxidation. The most feasible explanation seems to be that the deciding factor was the instability of the pyruvate oxidase system in the absence of pyruvate.

Some experimental points

The procedure should follow that of Kinnersley *et al.* [1935] (Appendix, p. 712), with the following modifications. The birds should be thoroughly depleted of vitamin by dosing them at least once after symptoms have appeared and using them upon the reappearance of symptoms. Only the cerebrum and

[1] There was a misprint on page 66 of this paper. In Table II after the arrow in the heading, it should read "poison" addition instead of "substrate" addition.

optic lobes are used. The bottles should contain Ringer-phosphate solution at *p*H 7·3 and 6 mg. sodium pyruvate before the introduction of the samples. Tests showed that it made no difference whether the pyruvate was added before or after the mashing; it is more convenient to add it before. Triplicate estimations are made if possible, and there should be an attempt to get the points 0, 0·25 and 0·5γ with pure vitamin. The zero point can be obtained in duplicate if only a few samples are available. After division with the glass crusher, which should be carefully done, the vitamin should be added to form the last addition to the bottle soon after the crushing. In this way the concentration is not even temporarily larger than is intended. After the addition of vitamin the bottles should be allowed to stand at room temperature for about 10 min. before filling them with O_2 with sufficient shaking to ensure that they do not become anaerobic. An extra shake after filling them with O_2 is desirable. I have found it convenient to place the bottles in the warm bath at 38° at intervals of two each $\frac{1}{2}$ min.; this allows reasonable time for reading. The calculations are made by plotting the average for the extra O_2 uptake on squared paper for the period 1–2 hr. and reading the value for the unknown by inspection. As a convention I have usually joined the points 0, 0·25 and 0·5γ by straight lines when the 0·25γ point has not lain upon the direct line between 0 and 0·5γ. In some cases it would be unquestionably correct to continue a straight line through the points 0 and 0·25γ and assume that this becomes discontinuous at some point between 0·25 and 0·5γ; but the possibility that there is more initial vitamin than the equivalent of 0·5γ in the tissue has to be balanced in such cases against the possible error of determining the points 0 and 0·25γ in question. The standard deviation of a single observation is of the order of 3·5% of the mean. It is not always easy to get more than ten satisfactory samples from a given brain; the mixing of two avitaminous brains has not proved an advantage owing to the increasing standard deviation found for individual samples. Hence the improvement with triplicate estimations must be balanced against the fewer determinations possible. The theoretical error for an estimation using triplicate observations is of the order of ±5%. It is difficult to give an exact idea of the error of the method in practice, because it is so much affected by the condition of the brain used; the results from some brains have to be discarded.

Exps. 1 and 2 below indicate the kind of results which may be expected and which are often obtained; but it must be emphasized that, as the method is subject to unaccountable variations, it is unwise to rely upon less than three estimations.

Exp. 1. Two tests were made in which 0·3γ of a vitamin solution was compared against 0·0, 0·25 and 0·5γ. The values found by estimation were 0·32 and 0·33γ.

Exp. 2. Two synthetic specimens of vitamin B_1, now known to be equally active, were tested at a level of 0·5γ. The mean O_2 uptakes during the period 1–2 hr. in duplicate estimations were in μl./g./hr.: specimen 1, with 0·0γ, 656 and with 0·5γ, 1027; specimen 2, with 0·5γ, 1063 and with 0·0γ, 654. The two separate values with 0·0γ agree well. The excess O_2 values of 371 and 409 give a value for specimen 2 of approx. 0·55γ. The extrapolation seems to be justifiable in this case; but the experiment was an early one, done before it was realized that 0·5γ was practically the maximum amount which could be estimated by this method.

The sodium pyruvate used was prepared as advised by Dr Stedman. I have found it best to dilute redistilled pyruvic acid with about an equal volume of water, add bromophenol blue and neutralize with concentrated pure NaOH to *p*H 4·5 approximately.

After cooling in ice-salt mixture, the salt is thrown out with acetone. Recrystallization was best done by dissolving in a minimum amount of water, treating with acetone until the appearance of strong turbidity, warming until the whole had just dissolved, and cooling in an ice-salt mixture. Purity was checked by estimation of the bisulphite-binding power.

Summary

The catatorulin test using avitaminous pigeon brain has been studied further; it may still occasionally be useful owing to its specificity for small amounts of vitamin B_1 (about $0{\cdot}2\gamma$).

Several improvements have been introduced; pyruvate is substituted for lactate. An explanation has now been found for the discontinuous type of curve found with pyruvate and the upper limits of sensitivity have been defined.

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REFERENCES

Jansen & Westenbrink (1938). See Westenbrink & Goudsmit, *Nature, Lond.*, **142**, 151.
Kinnersley, O'Brien & Peters (1935). *Biochem. J.* **29**, 701.
McGowan & Peters (1937). *Biochem. J.* **31**, 1637.
Ochoa & Peters (1938). *Biochem. J.* **32** (in the Press).
Ogston & Peters (1936). *Biochem. J.* **30**, 736.
Passmore, Peters & Sinclair (1933). *Biochem. J.* **27**, 842.
Peters (1936). *Biochem. J.* **30**, 2206.
—— (1937). *Biochem. J.* **31**, 2240.
—— Rydin & Thompson (1935). *Biochem. J.* **29**, 57 and 63.
Sinclair (1937). *Nature, Lond.*, **140**, 361.
Westenbrink & Polak (1937). *Rec. trav. chim.* **56**, 315.

CCLXIV. THE SUGAR AND TOTAL KETONE CONTENT OF THE BLOOD OF EWES AND OF THEIR NEW-BORN LAMBS

By LAURENCE C. SNOOK and WILLIAM GODDEN

From the Rowett Research Institute, Aberdeen

(*Received 26 September 1938*)

In many animals it has been found that the maternal blood at term contains a higher percentage of sugar than the foetal blood. Needham [1931] cites data illustrating this point and gives figures obtained in human subjects by 18 investigators, from which the following averages have been calculated: maternal blood 118 mg., foetal blood 94 mg. sugar per 100 ml. Aron [1924] records even wider differences for the dog (maternal 110 mg., foetal 65 mg. per 100 ml.) and for the guinea pig (maternal 107 mg., foetal 60 mg. per 100 ml.). On the other hand he gives data for the cow (maternal 100 mg., foetal 110 mg.) and for the pig (maternal 100 mg., foetal 139 mg.) which suggest that the reverse is true for these species. Passmore & Schlossman [1938] record a similar relation for sheep and goats.

In the course of a study on the occurrence of ketonaemia in pregnant ewes the opportunity was taken to determine the sugar and total ketones present in the blood of ewes (both normal and ketonaemic) at the time of parturition and in that of their new born lambs. A number of ewes were available which, for some weeks during the latter half of pregnancy, had shown progressively increasing ketonaemia accompanied by marked hypoglycaemia. The lambs from these ewes were small and weak and it was of interest to see whether this weakness could be correlated with a low blood sugar level and/or the passage of ketones from the maternal to the foetal blood stream. As controls a number of healthy ewes with their vigorous new-born lambs were also examined.

Methods

Blood sugar: Somogyi's modification of the Shaffer-Hartmann method [Peters & Van Slyke, 1931].

Total ketone bodies in the blood: Gravimetric method using Denigès' reagent [Peters & Van Slyke, 1931].

Results

All the ewes were bled a day or more prior to parturition and as soon after lambing as possible, the new-born lambs being bled at the latter time. The analytical data are recorded in Table I with the times before and after lambing at which the samples were taken. They serve to show the changes which occur in the blood of the ewe at about the time of labour as well as the differences between the blood of the new-born lamb and its dam with respect to sugar and ketone bodies.

Blood sugar. In the normal healthy ewe at parturition there is a marked rise in the blood sugar level, but the figures cited suggest that *post partum* there is a fairly rapid return to the normal level. Thus, although the ewes bled up to within

Table I. *The sugar and total ketone bodies in the blood of ewes at parturition and in that of their new-born lambs: mg. per 100 ml.*

Ewe no.	Days before lambing	Ewe: Before lambing Sugar	Ewe: Before lambing Ketones	Ewe: After lambing Sugar	Ewe: After lambing Ketones	Lamb Sugar	Lamb Ketones	Interval between lambing and bleeding
				Healthy ewes				
40	1	52	0	145	0	163	0	10 min.
65	6	56	0	112	0	156	0	30 ,,
68	2	60	0	83	0	109	0	2½ hr.
44	1	56	0	72	0	94	0	3 ,,
79	3	50	0	50	0	97	0	14 ,,
				Ewes showing ketonaemia				
81	3	18	48	62	25	62	0	5 min.
48	1	40	36	55	16	65	0	5 ,,
41	8	32	41	—	44	—	0	5 ,,
4	1	31	83	67	60	124	0	15 ,,
89	4	38	12	46	12	64	0	15 ,,
37	6 hr.	39	28	37	24	92	0	30 ,,
98	6	28	48	64	32	93	0	60 ,,
11	8	28	53	53	33	68	0	60 ,,

3 hr. after lambing showed this rise, in the case of ewe no. 79 which was not bled until 14 hr. after lambing, the blood sugar was at the same level as it had been 3 days before lambing. A similar rise in blood sugar level at term has been reported in the case of human subjects by Ketteringham & Austin [1938] who found that at the first stage of labour (1–8 hr. before delivery) 28 mothers had an average blood sugar of 93 mg. per 100 ml. and at delivery the average value was 122 mg. per 100 ml. In the ewes suffering from ketonaemia and having a low blood sugar level before lambing the rise was also shown but the level reached was by no means as high as in the healthy ewes.

In the case of the lambs it is shown that at birth, or shortly after, their blood contained a higher percentage of sugar than did that of the mother. This difference is pronounced in the case of the healthy ewes and their progeny, but is less marked for the lambs from some of the ketonaemic ewes. It is probable that, following birth, the blood sugar levels of the lamb may change with rapidity over a fairly wide range. Thus Passmore & Schlossman [1938] found a rise in the blood sugar from 128 to 142 mg. per 100 ml. in the 15 min. following the delivery of an experimental lamb which had been treated with insulin. They suggest a *post partum* mobilization of liver glycogen as a result of the exposure of the new-born lamb to a cold atmosphere. If this elevation is general the rather low blood sugar levels obtained 5 min. after birth could be explained and the rather higher values obtained soon after might be expected. Ketteringham & Austin [1938] found that 3–6 hr. after birth the blood sugar of human babies dropped to the normal infant range. The results shown for the lambs from normal ewes suggested that an analogous change could be demonstrated in the lamb. The smaller differences between the blood sugar levels of the ketonaemic ewes and their lambs and the smaller changes in the blood sugar level of these lambs at the longer intervals from the time of birth are in all probability attributable to the hypoglycaemic condition of the ewe during the latter stages of pregnancy.

Blood ketones. An interesting finding of this investigation is the complete absence of ketone bodies from the blood of the new-born lambs from ewes which had been showing signs of severe ketonaemia for periods up to 6 weeks prior to and immediately after lambing. One might expect that molecules of the size

and nature of β-hydroxybutyric acid (the chief ketone present in the blood of the ketonaemic ewe) would pass readily through the placental membrane. Many investigators have shown that in most mammals gases and crystalloid substances with small molecules pass readily through the placenta. Thus Anselmino [1929] found that 50 min. after injecting dihydroxyacetone into the blood stream of the pregnant rabbit, the foetal and maternal bloods contained the same concentration of this added material. Needham [1931] discusses the variation in permeability shown by different types of chorion and gives as an illustration the fact that sodium salicylate will pass readily through the placenta of the cat or the guinea-pig but not through that of the cow or ewe. It seems that the placenta of the ewe likewise prevents the passage of ketone bodies to the foetus.

It is possible that the foetal lamb possesses some mechanism facilitating the oxidation or removal of ketone bodies from the system, but this does not seem probable. It is generally accepted that young animals develop ketosis more readily than adults and suffer more severely from its effects. This does not suggest the existence of any special protective mechanism in the young.

Summary

1. The sugar and total ketones in the blood of normal and ketonaemic ewes prior to and shortly after lambing are recorded along with the corresponding figures for the blood of the lamb, drawn shortly after birth.

2. At parturition there is a marked rise in the blood sugar levels of both normal and hypoglycaemic ewes.

3. The blood of the new-born lamb contains a higher percentage of sugar than that of its dam bled at the same time.

4. Hypoglycaemia in the pregnant ewe is reflected in a much lower blood sugar level at parturition and a relatively low blood sugar level in the new-born lamb.

5. The blood of new-born lambs from ewes showing marked ketonaemia was quite free from ketone bodies.

REFERENCES

Anselmino (1929). *Arch. Gynaek.* **138**, 710.
Aron (1924). *Arch. int. Physiol.* **22**, 273.
Ketteringham & Austin (1938). *Amer. J. med. Sci.* **195**, 318.
Needham (1931). "Chemical Embryology." University Press, Cambridge.
Passmore & Schlossman (1938). *J. Physiol.* **92**, 459.
Peters & Van Slyke (1931). "Quantitative Clinical Chemistry." Baillière, Tindall & Cox, London.

CCLXV. THE SPECIFICITY OF COUPLED ESTERIFICATION OF PHOSPHATE IN MUSCLE

By DOROTHY MOYLE NEEDHAM and GWEI DJEN LU

From the Biochemical Laboratory, Cambridge

(*Received 1 October 1938*)

It has been shown that, while a number of oxidoreductions involving oxidation of triosephosphate to phosphoglyceric acid can be coupled with synthesis of adenylpyrophosphate from adenylic acid and free phosphate, similar oxidoreductions involving oxidation of glyceraldehyde to glyceric acid are not so coupled [Needham & Pillai, 1937]. Further experiments, in which a variety of conditions have been tried, have confirmed this lack of coupling. From these observations the inference may be drawn that the essential energy-providing reaction in the coupled mechanism is the reduction of coenzyme I by glyceraldehydephosphate; for the oxidation of reduced coenzyme by pyruvate goes on (when glyceraldehyde is the reductant in the oxidoreduction) without coupled esterification. As this inference must be important in the elucidation of the mechanism of the coupling, it was decided to test it further.

This has been done by separating the oxidoreduction into its two constituent reactions:

(1) glyceraldehydephosphate + coenzyme → phosphoglyceric acid + reduced coenzyme,

(2) pyruvate + reduced coenzyme → lactic acid + coenzyme,

and testing each separately for its effect on esterification. The experiments with reaction (2) are quite straightforward, but with reaction (1) a complication arises. In muscle extract it has not so far been possible to separate the glycerophosphate dehydrogenase from the triosephosphate dehydrogenase; this means that, in aqueous muscle extracts or in extracts of acetone powder, triosephosphate acts as oxidant as well as reductant, and the coenzyme reduced during phosphoglyceric acid formation is oxidized by more triosephosphate, which is itself reduced to glycerophosphate. We have therefore studied the triosephosphate oxidation to phosphoglycerate under conditions where the reduced coenzyme was largely, if not entirely, reoxidized by atmospheric O_2 through the cytochrome system. To enable O_2 uptake to go on, it was necessary to add to the extract of muscle acetone powder ordinarily used for study of oxidoreductions, a small amount of an enzyme preparation from heart made according to the method of Keilin & Hartree [1938]. This preparation contains (1) coenzyme factor, the enzyme catalysing the transfer of hydrogen from reduced coenzyme I or II to a carrier such as cytochrome [Dewan & Green, 1938]; (2) cytochromes *a* and *b*; (3) cytochrome oxidase, the enzyme catalysing the oxidation of reduced cytochrome by atmospheric O_2 [Keilin & Hartree, 1938]. When this heart

preparation is added to muscle extract, the following series of reactions can take place:

$$\text{Triosephosphate} + \text{coenzyme I} \xrightarrow[\text{dehydrogenase}]{} \text{phosphoglycerate} + \text{reduced coenzyme.}$$

$$\text{Reduced coenzyme} + \text{cytochrome} \xrightarrow[\text{factor}]{\text{coenzyme}} \text{coenzyme} + \text{reduced cytochrome.}$$

$$\text{Reduced cytochrome} + O_2 \xrightarrow[\text{oxidase}]{\text{cytochrome}} \text{cytochrome.}$$

The amount of cytochrome present in our preparations was not optimal, and greater O_2 uptake was always obtained by adding methylene blue. Then

$$\text{Reduced coenzyme} + \text{MeB} \xrightarrow{\text{coenzyme factor}} \text{coenzyme} + \text{reduced MeB.}$$

$$\text{Reduced MeB} + O_2 \longrightarrow \text{MeB.}$$

Experimental details

Enzyme preparations. The aqueous extract of dried acetone muscle powder (used in all the experiments to provide the oxidoreduction and phosphorylation mechanisms) was prepared in the same way as described by Needham & Pillai [1937]. Before use the extracts were dialysed 18 hr.

The heart preparation (added when interaction with atmospheric O_2 was needed) was made from pig's heart in the following way. Two hearts were freed from fat and minced twice through an ordinary mincer; the mince was thoroughly washed by suspending (with mechanical stirring) in about 5 l. tap water; this was repeated ten times, the muscle mass being squeezed in muslin each time before resuspension. It was then ground with fine sand and 250 ml. $M/25$ phosphate buffer (pH 7) for $1\frac{1}{2}$ hr. in a mechanical mortar. The thick paste was mixed with 350 ml. more buffer solution and centrifuged. The cloudy fluid was poured off, and acetate buffer pH 4·6 was added until the pH of the whole had fallen to 4·6. The mixture was again centrifuged; the clear fluid was discarded, and the residue was suspended in 30 ml. phosphate buffer ($M/10$ pH 7·2). Finely powdered Na_2HPO_4 was added until the pH was 7·0. The preparation was stored at 0° in this form; at the end of a week its activity was about 60% of the initial. Before use, portions of the preparation were dialysed 18 hr. in cellophane bags suspended in 3 l. 0·5% KCl.

It was already known that the extract of acetone muscle powder contained no adenylpyrophosphatase and no deaminase. As the heart preparation also was to be used in experiments where adenylpyrophosphate synthesis from adenylic acid was to be looked for, it was necessary to find out in this case too whether enzymes were present which could dephosphorylate synthesized adenylpyrophosphate, or deaminate added adenylic acid.

As regards possible destruction of added adenylic acid, the position appeared satisfactory; for under the conditions of the experiments, not more than 5% of the possible NH_3 was set free in 15 min.

On the other hand, unfortunately, it seemed to be impossible to free the heart preparation from adenylpyrophosphatase. Even after some days at 0° and several hr. dialysis added adenylpyrophosphate was broken down to a considerable extent. In order in these circumstances to demonstrate disappearance of inorganic phosphate, we tried adding to the experimental mixture glycogen as well as adenylic acid, in the hope that phosphate would be transferred to glycogen from synthesized adenylpyrophosphate more rapidly than it was split

off again from the adenylpyrophosphate to give free phosphate. Experiments in which adenylpyrophosphate + glycogen were added to the enzyme mixture showed that, even in the presence of glycogen, rather more than half the pyrophosphate disappearing was found as inorganic phosphate; thus the adenylpyrophosphatase appeared to be more active than the enzyme concerned with phosphate transfer. Creatine (at *p*H 9·0) instead of glycogen was tried, but with even less success. In absence, therefore, of any better way of demonstrating esterification of free phosphate, glycogen + adenylic acid were used in the O_2 uptake experiments.

The use of glycogen in this way led to the possibility of another source of error: the Parnas esterification of inorganic phosphate with glycogen might take place, leading to disappearance of inorganic phosphate quite independently of the coupled mechanism. Tests such as the following showed, however, that with the concentrations of phosphate, glycogen and enzymes used in the experiments, no Parnas esterification was observable.

Three mixtures were prepared. Each contained glycogen 3·6 mg., adenylic acid 0·6 mg., phosphate (0·066 *M*, *p*H 7·2) 0·3 ml., $MgSO_4$ (0·006 *M*) 0·05 ml. and NaF (0·5 *M*) 0·1 ml.; water to 2·4 ml. In addition the following were added:

	1	2	3
Heart preparation	0·75 ml.	0·75 ml.	0 ml.
Muscle extract	0·25 ml.	0 ml.	0·25 ml.

An initial sample of 0·5 ml. was removed from each, then all were incubated at 37°. Samples were again removed at the end of 15 min. and 30 min. Only in no. 3 was any change in free phosphate observed, and here the decrease was only 0·09 mg. out of 0·6 mg.

Finally, tests were made to see whether the hexosediphosphatase activity discussed by Pillai [1938, 1] was of importance under our conditions.

Three mixtures were prepared and incubated at 37°. Each contained hexosediphosphate (0·14 *M*) 0·15 ml., phosphate (0·066 *M*, *p*H 7·2) 0·3 ml., $MgSO_4$ (0·006 *M*) 0·1 ml. and NaF (0·5 *M*) 0·1 ml.; water to 2·2 ml. In addition the following were added:

	1	2	3
Heart preparation	0·75 ml.	0·75 ml.	0 ml.
Muscle extract	0·25 ml.	0 ml.	0·25 ml.

Samples were removed at 0, 15 and 30 min. The increase in inorganic phosphate found was within the limits of experimental error.

Summing up this preliminary examination of the properties of the enzyme preparations to be used, we may therefore say that no destruction of adenylic acid by deaminase, no significant removal of free phosphate by the Parnas esterification, and no significant increase in free phosphate by hexosediphosphatase activity is to be expected. On the other hand, owing to the persistence of adenylpyrophosphatase in the heart preparation, some hydrolysis of adenylpyrophosphate, even in presence of glycogen, will take place, and one can hope to observe only a fraction of the adenylpyrophosphate synthesis actually occurring.

Chemical preparations and methods. These were for the most part the same as those used in previous work [e.g. Needham & Pillai, 1937]. For estimation of phosphoglyceric acid the method of Rapoport [1937] was followed and for O_2 uptake the method of Warburg, using 15 ml. conical cups with two side-bulbs.

Lack of esterification with glyceraldehyde oxidoreduction

In the preliminary experiments of Needham & Pillai [1937] it was found that oxidoreduction between glyceraldehyde and pyruvate went on unaccompanied by esterification of free phosphate when adenylic acid was present as phosphate acceptor. About 1·5 mg. lactic acid per ml. extract were formed; when similar amounts of lactic acid were formed by reduction of pyruvate by triosephosphate, about 0·5 mg. inorganic P disappeared. In the present work these results were confirmed. It seemed possible that the high adenylic acid concentration used (about 3 mg./ml. final concentration) might have some deleterious effect upon the coupled mechanism with glyceraldehyde as a component, although not with triosephosphate. Some experiments were therefore done with glycogen and creatine provided as possible acceptors of phosphate from adenylpyrophosphate if formed, only small amounts of adenylic acid being added.

Typical experiments are given below.

1 ml. extract was made up to a final volume of 3 ml. The final concentrations were: pyruvate, 0·026 *M*; hexosediphosphate, 0·013 *M*; inactive glyceraldehyde, 0·052 *M* (to allow for the *l*-component, which does not react); phosphate, 0·03 *M*; NaF, 0·025 *M*; $NaHCO_3$, 0·01 *M*. Crude coenzyme was added, 0·3 ml. of a solution containing 150 mg. per 100 ml. All samples were incubated 30 min. at 37°, *p*H 8·0. The results are expressed in mg. per ml. extract.

Table I

	Lactic acid mg.	Inorganic P mg.	Creatine-phosphate P mg.
Adenylic acid, 0·01 *M* (3·4 mg. per ml.)			
Hexosediphosphate + pyruvate	+1·2	−0·5	—
Glyceraldehyde + pyruvate	+1·5	0	—
Adenylic acid, 0·002 *M* (0·7 mg. per ml.)			
Hexosediphosphate + pyruvate + glycogen (6 mg.)	+0·94	−0·27	—
Glyceraldehyde + pyruvate + glycogen (6 mg.)	+0·90	0	—
Hexosediphosphate + pyruvate + creatine (10 mg.)	+1·31	—	+0·17
Glyceraldehyde + pyruvate + creatine (10 mg.)	+1·10	—	0

In the case of glyceraldehyde oxidoreduction with pyruvate, it was noticed that the lactic acid formation was much greater in a sample to which no adenylic acid or phosphate had been added. Further experiment showed that addition of adenylic acid has a marked inhibitory effect on the oxidoreduction, while addition of phosphate has a stimulating effect.

Table II

	Lactic acid formed (mg.)	
	Exp. 45 A	Exp. 45 B
No adenylic acid and no phosphate added	0·43	0·37
Both added	0·84	1·05
Phosphate added, no adenylic acid	1·44	1·24

For hexosediphosphate oxidoreduction with pyruvate the following results were found:

Table III

	Lactic acid formed (mg.)		
	Exp. 46	Exp. 48	Exp. 59
No adenylic acid and no phosphate added	0·84	0·64	—
Both added	1·28	2·4	1·19
Phosphate added, no adenylic acid	1·09	1·54	0·39

Here again the stimulating effect of phosphate addition is seen, but in this case adenylic acid shares in the stimulation instead of opposing it.

In the previous work of Needham & Pillai [1937] a stimulatory effect of higher adenylic acid concentrations (0·02 and 0·03 *M*) upon the oxidoreduction with triosephosphate was noticed; and Pillai [1938, 2] has found that with very thoroughly dialysed extracts (90 hr.), though oxidoreduction can go on without addition of phosphate or adenylic acid, activation follows on addition of these substances. Meyerhof *et al.* [1937; 1938] have observed the very marked stimulating effect of adenosinediphosphate upon reduction of coenzyme I by triosephosphate in presence of B protein from yeast, and this has been confirmed by Adler & Günther [1938]. Meyerhof and his collaborators consider that only when the esterification process is going on can the oxidoreduction between triosephosphate and pyruvate proceed. In view of all these facts the inhibitory effect of adenylic acid upon the uncoupled glyceraldehyde oxidoreduction is interesting.

Esterification accompanying triosephosphate oxidation

In these experiments 1 ml. heart preparation and 0·3 ml. acetone powder extract were used in each Warburg cup, with 0·1 ml. 5% methylene blue. The amount of heart preparation was the limiting factor in the O_2 uptake, which was not increased by using more muscle extract or carrier. The total volume was 3–4 ml. The concentration of phosphate was about 0·01 *M* and its *p*H 7·2. Crude coenzyme I was added, 0·4 ml. of a solution containing 150 mg. per 100 ml. NaF 0·025 *M* was always present to prevent breakdown of the phosphoglyceric acid formed. As explained in an earlier section, adenylic acid was used in low concentration as transporter in these experiments, and glycogen was present to accept the phosphate. About 1 mg. adenylic acid and 6 mg. glycogen were present in each cup. To provide the triosephosphate for oxidation, hexosediphosphate was added in amount to give a final concentration of 0·01 *M*. The neutralized solutions of hexosediphosphate and of adenylic acid were placed in the two side-bulbs, and were tipped in only after temperature equilibration.

Controls showed that (*a*) without added hexosediphosphate no O_2 uptake occurred; (*b*) addition of methylene blue increased the O_2 uptake by about 50%; (*c*) it was very low without acetone powder extract; (*d*) with acetone powder extract but no heart preparation it was about 20% of the amount when both were present. Iodoacetate (0·003 *M*) completely inhibited the O_2 uptake with hexosediphosphate.

All samples in Table IV were kept 30 min. at 37°; the results are given in μl. and mg. per cup.

In most of these experiments, no dismutation of the hexosediphosphate has taken place. This is shown by the fact that the increase in difficultly hydrolysable P and the increase in phosphoglyceric acid P are about equivalent to each other and to the O_2 uptake. In a minority of the experiments, however, dismutation as well as oxidation of the triosephosphate took place. This is shown by the fact that the increase in difficultly hydrolysable P is much more than the equivalent of the O_2 uptake. In Exp. 30, for example, where the phosphoglyceric acid P was measured as well, this was found to be greater than the P equivalent of the O_2 uptake by 0·5 mg. This amount therefore must have been formed by dismutation, and the presence of 0·5 mg. glycerophosphate P was to be expected. 2·24 mg. difficultly hydrolysable P were indeed found.

Table IV

Exp.	O_2 uptake µl.	P equiv. of O_2 uptake mg.	Increase in diff. hydr. P mg.	Phospho-glyceric acid P formed mg.	Change in inorganic P mg.
24	210	0·6	0·52	—	−0·7
25	153	0·43	0·55	—	−0·66
27	468	1·3	1·7	—	−0·71
27	384	1·1	1·41	—	−0·89
31	231	0·64	0·52	0·748	−0·20
31	198	0·55	0·70	0·746	−0·21
31	249	0·68	0·72	0·702	−0·29
32	172	0·48	0·43	0·62	−0·19
32	181	0·50	0·64	0·71	−0·22
35	252	0·73	0·82	1·00	−0·46
37	335	0·93	0·87	1·1	−0·25
37	310	0·86	0·97	1·1	−0·22
37	300	0·84	0·99	1·1	−0·30
64	267	0·74	0·63	—	−0·4
64	429	1·2	0·99	—	−0·75
26	380	1·13	2·205	—	−1·02
26	263	0·77	2·17	—	−0·98
30	389	1·07	2·24	1·54	−0·71

These results on muscle may be compared with those obtained by Lennerstrand & Runnström [1935] and Lennerstrand [1936] on apozymase from dried yeast. Adenylpyrophosphatase seems to be absent from such preparations, for there was good agreement between inorganic phosphate disappearing and phosphoglyceric acid formed. Lennerstrand found that, using phosphate concentrations of about 0·03 *M*, the O_2 uptake was much less than with higher P concentrations. At a P concentration of nearly 0·3 *M* it had increased to tenfold. This effect was caused by long persistence of the power of O_2 uptake, the initial rate being about the same at all P concentrations. No correspondingly large effects were observed with muscle. Raising the P concentration from 0·01 to 0·03 *M* caused a 50% rise in O_2 uptake; but further increase to 0·1 *M* caused no further rise.

Meyerhof *et al.* [1938], using "B protein" from yeast (which contains no glycerophosphate dehydrogenase), have recently shown that when triose-phosphate reacts with coenzyme I to form phosphoglyceric acid and reduced coenzyme there is equivalent phosphate esterification.

Some experiments were done in which lactate and succinate were added as substrate for oxidation. The experimental conditions were as usual, except that, with lactate, HCN was added to remove the pyruvate formed [Green & Brosteaux, 1936]. No esterification accompanied O_2 uptake in these cases.

Table V

Substrate	O_2 uptake µl.	Change in inorg. P mg.
Lactate	190	−0·06
Succinate	240	−0·10

Lack of esterification with oxidation of reduced coenzyme

In these experiments excess of pyruvate together with a known amount of reduced coenzyme I were added to the extract of muscle acetone powder, in presence of phosphate and adenylic acid. The amounts of reduced coenzyme I, of lactic acid and of inorganic P were estimated at the beginning and end of the incubation time.

The estimation of reduced coenzyme was made by measurement of the height of the absorption curve at 345 mμ, using a Spekker photometer.

The absorption coefficient $\beta = \frac{1}{d} \times \frac{1}{c} \times \ln \frac{I_0}{I}$,

where d = length of tube in cm.

c = concentration of absorbing solute in g. mol. per ml.

$\frac{I_0}{I} = \frac{\text{intensity of light entering tube}}{\text{intensity of light leaving tube}}$.

Warburg *et al.* [1935; 1938] have shown for the dihydro bands at 345 mμ, characteristic of the nucleotides coenzymes I and II, that

$$\ln \frac{I_0}{I} = 1{\cdot}3 \times 10^7,$$

when $d = 1$ and $c = 1$ g. mol. per ml. When $d = 2$ (as in our experiments) and $c = 0{\cdot}1$ mg. per ml.

$$\ln \frac{I_0}{I} = \frac{1{\cdot}3 \times 10^7 \times 2}{663 \times 10{,}000}.$$

As the drum readings on the Spekker instrument are engraved to give $\log \frac{I_0}{I}$, not $\ln \frac{I_0}{I}$, the reading to be expected for 0·1 mg. reduced coenzyme I per ml. in the 2 cm. tube is

$$\log \frac{I_0}{I} = \frac{1{\cdot}3 \times 10^7 \times 2}{663 \times 10{,}000} \times \frac{1}{2{\cdot}3} = 1{\cdot}7.$$

Solutions of coenzyme I of unknown concentration were diluted to give a reading of about this value, and the original concentration could then be calculated.

The coenzyme I preparation used was made in the usual way [see Needham & Pillai, 1937]. Tests showed that it contained about 25% of the coenzyme. The reduced coenzyme was prepared from it by treatment with $Na_2S_2O_4$, excess $Na_2S_2O_4$ was oxidized by vigorous aeration and the Na_2SO_3 was removed according to the method of Euler & Hellström [1938]. One experiment is described in detail below.

300 mg. crude coenzyme and 40 mg. $Na_2S_2O_4$ were dissolved in 6·6 ml. 1·3% $NaHCO_3$ which had previously been gassed with a stream of N_2+5% CO_2. The mixture stood at room temp. 1 hr. with the gas stream running. The N_2-CO_2 stream was then exchanged for an air current, which ran for 2 hr. At the end of this time, the mixture was warmed to 80°, a slight excess of $BaCl_2$ was added and it was filtered through a heated funnel. The excess Ba was removed with Na_2SO_4. The final volume was 8·4 ml. Two mixtures were made up in Warburg cups, which were then filled with N_2-5% CO_2 mixture and shaken at 18°, until the gas absorption (due to the decrease in acidity when reduced coenzyme is oxidized) was finished.

Each contained reduced coenzyme solution 2 ml., adenylic acid (0·1 *M*) 0·2 ml., phosphate (0·05 *M*, *p*H 7·2) 0·5 ml., $NaHCO_3$ (3·3%) 0·4 ml. and muscle extract 0·3 ml.; to cup no. 1 pyruvate (0·25 *M*) 0·2 ml. was also added, while 0·2 ml. water was added to cup no. 2.

At the end of the time the cups were emptied into test tubes and washed out with three successive lots of 2 ml. water. The contents of the tubes were coagulated by placing in a boiling water bath and were then mixed with a small quantity of kieselguhr (*ca.* 20 mg.) and filtered through paper. The volume of each was made up to 20 ml. 2 ml. were used for phosphate determination, 15 ml. for lactic acid and 1 ml. was diluted to 5 ml. for measurement of the absorption spectra (Table VI).

Table VI

	Reduced coenzyme mg.	Lactic acid mg.	Phosphate mg.
1	0	1·83	0·87
2	9·4	0·69	0·87

Thus 1·14 mg. lactic acid have been formed from pyruvate, while 9·4 mg. reduced coenzyme (equivalent to 1·25 mg. lactic acid) have been oxidized. The inorganic phosphate remained absolutely unchanged.

In order to make sure that no substance capable of preventing phosphorylation was present in the reduced coenzyme solution used, the following control experiment was carried out. The "control solution" was made by carrying out all the processes involved in reduction of coenzyme, and removal of the $Na_2S_2O_4$ and Na_2SO_3, but without adding coenzyme. Three mixtures were made up. Each contained hexosediphosphate (0·14 *M*) 0·2 ml., pyruvate (0·25 *M*) 0·2 ml., adenylic acid (0·1 *M*) 0·2 ml., phosphate (0·066 *M*) 0·5 ml., $NaHCO_3$ (1·3 %) 1·0 ml., NaF (*M*) 0·1 ml., coenzyme solution (0·15 %) 0·2 ml. and muscle extract 0·3 ml. No. 1 contained also 2·0 ml. water, nos. 2 and 3 contained each 2·0 ml. control solution. Nos. 1 and 2 were incubated 10 min. at 37°; then coagulated by placing in boiling water. In the case of No. 3, the tube was placed in boiling water before addition of the enzyme solution, so that the latter was coagulated at once. All were mixed with kieselguhr and filtered as usual, and the inorganic P was estimated (Table VII).

Table VII

No.	Inorganic P mg.	P esterified mg.
1	0·55	0·22
2	0·52	0·25
3	0·77	—

The control solution has thus had no inhibitory effect upon the phosphorylation brought about by oxidoreduction between triosephosphate and pyruvate.

Meyerhof *et al.* [1938] reached the same conclusion that, in yeast, oxidation of reduced coenzyme by acetaldehyde is not connected with phosphorylation. In their experiments the phosphate disappearance was equivalent to the phosphoglyceric acid formed, as already mentioned, and this was the case whether or not acetaldehyde was introduced to reoxidize the reduced coenzyme.

At the beginning of this work the attempt was made to use the reduced coenzyme solution for experiments like that in Table VI without removing the Na_2SO_3 formed on aeration. But experiments on the lines of Table VII showed that these "control solutions" containing Na_2SO_3 had a very marked inhibitory effect upon phosphorylation brought about by oxidoreduction between triosephosphate and pyruvate; the actual oxidoreduction, as measured by lactic acid formation, was unaffected.

Table VIII shows a comparison of the effects of the "control solution" (10 mg. $Na_2S_2O_4$ in 8 ml. 0·1 % $NaHCO_3$; well aerated), Na_2SO_3 solution (0·014 *M*) and arsenate solution (0·014 *M*). All the samples contained coenzyme, pyruvate, hexosediphosphate, adenylic acid, phosphate, muscle extract and NaF as in the experiment of Table VII.

Table VIII

	Lactic acid formed mg.	P esterified mg.
1. +3 ml. 1 % $NaHCO_3$	1·2	0·35
2. +3 ml. control solution	0·94	0·19
3. +3 ml. 0·02 *M* arsenate in 1 % $NaHCO_3$	1·50	0
4. +3 ml. 0·02 *M* Na_2SO_3 in 1 % $NaHCO_3$	1·09	0·19

This effect of sulphite is being further investigated.

SUMMARY

1. Further evidence has been obtained showing that in muscle extract oxidoreductions involving glyceraldehyde are not accompanied by coupled esterification of phosphate.

2. The oxidoreduction between triosephosphate and pyruvate has been separated into its two constituent reactions. The oxidation of triosephosphate to phosphoglyceric acid by coenzyme is accompanied by esterification; the oxidation of reduced coenzyme thus formed by pyruvate is not accompanied by esterification.

3. Adenylic acid has an activating effect on oxidoreduction involving triosephosphate, but an inhibitory effect on oxidoreduction involving glyceraldehyde.

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REFERENCES

Adler & Günther (1938). *Hoppe-Seyl. Z.* **253**, 143.
Dewan & Green (1938). *Biochem. J.* **32**, 626.
Euler & Hellström (1938). *Hoppe-Seyl. Z.* **252**, 31.
Green & Brosteaux (1936). *Biochem. J.* **30**, 1489.
Keilin & Hartree (1938). *Proc. roy. Soc.* B, **125**, 171.
Lennerstrand (1936). *Biochem. Z.* **289**, 104.
—— & Runnström (1935). *Biochem. Z.* **283**, 12.
Meyerhof, Ohlmeyer & Möhle (1938). *Biochem. Z.* **297**, 90.
—— Schulz & Schuster (1937). *Biochem. Z.* **293**, 309.
Needham & Pillai (1937). *Biochem. J.* **31**, 1837.
Pillai (1938, 1). *Biochem. J.* **32**, 1087.
—— (1938, 2). *Biochem. J.* **32**, 1961.
Rapoport (1937). *Biochem. Z.* **291**, 429.
Warburg (1938). *Ergebn. Enzymforsch.* **7**, 210.
—— Christian & Griese (1935). *Biochem. Z.* **282**, 157.

CCLXVI. THE CATALYTIC EFFECT OF BUFFERS ON THE REACTION $CO_2+H_2O \rightleftharpoons H_2CO_3$

By F. J. W. ROUGHTON and V. H. BOOTH[1]

From the Physiological Laboratory, Cambridge

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FAURHOLT [1925, 1] has shown that in aqueous solution CO_2 takes part in two independent reactions

$$CO_2+H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-, \quad \text{.....(1)}$$

$$CO_2+OH^- \rightleftharpoons HCO_3^-. \quad \text{.....(2)}$$

The velocity constant of the reaction $CO_2+OH^- \rightarrow HCO_3^-$ is more than a million times greater than the velocity constant of the reaction $CO_2+H_2O \rightarrow H_2CO_3$, the loss of the proton from the H_2O molecule apparently causing, as Faurholt has emphasized, an enormous increase in the affinity for CO_2. In consequence of this, it is only below *p*H 8 that the rate of (2) becomes negligible in comparison with the rate of (1): in the range *p*H 9–10 the two rates are of the same order, whilst above *p*H 10 the rate of (2) becomes predominant.

Similar studies on the rate of combination of CO_2 with very weakly acidic organic hydroxides, such as methyl alcohol, ethyl alcohol and glucose, have shown [Faurholt, 1927] that CO_2 reacts slowly and in an analogous way with the organic hydroxide molecule:

$$CO_2+HOX \rightleftharpoons HCO_3X. \quad \text{.....(3)}$$

The inhibitory effect of the proton is again shown by the fact that the reaction with the organic anion—

$$CO_2+OX^- \rightleftharpoons CO_3X^- \quad \text{.....(4)}$$

—is also very much more rapid and complete than the reaction with the molecule.

In the case of stronger oxy-acids the affinity of the anion for H^+ is correspondingly lower, and Faurholt tacitly assumed that the affinity of the anion for CO_2 would fall *pari passu* and hence that acids with *pK* of the order of 7 (e.g. cacodylic acid) could be safely used as buffers in the measurements of the velocity constants of the reactions $CO_2+H_2O \rightleftharpoons H_2CO_3$ and $CO_2+OH^- \rightleftharpoons HCO_3^-$, without fear of the buffer taking any part other than the "instantaneous" supply or removal of the H^+ ions involved in the ionization of carbonic acid, i.e. $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$. The possibility of any direct reaction of the buffer with CO_2, H_2CO_3 or HCO_3^- (except in the case of the borate ion, which Faurholt suggested might form some special complex with CO_2) was thus implicitly excluded, not only by Faurholt but by all others who have worked in this field.

Preliminary experiments on the activity of carbonic anhydrase in phosphate solutions of varying concentration and *p*H have, however, prompted us to examine more critically the role of the buffer in reactions (1) and (2). To this end we have measured manometrically both the rate of CO_2 uptake by buffer solutions shaken violently with CO_2, and the rate of CO_2 output from bicarbonate buffer mixtures, in the presence of a much wider range of buffer concentration

[1] George Henry Lewes Student.

and species than heretofore. Our results, especially those in which the buffer concentration is varied 10-fold or more, lead us to conclude that almost all common oxy-acid-salt mixtures which buffer above *p*H 6, not only promote the CO_2 reactions by supplying or removing H^+ ions, but also through their more negative constituents catalyse directly both phases of the reaction $CO_2+H_2O \rightleftharpoons H_2CO_3$. A preliminary account of these results has already appeared [Booth & Roughton, 1938]. Faurholt's discovery of the activity of the borate ion is thus only a special, though pronounced, example of a general principle.

It was not until nearly the end of the research that we investigated the effect of nitrogen bases as buffers. We were deterred from doing so by the fact that straight-chain nitrogenous bases, such as NH_3 or $CH_2NH_2COO^-$, combine rapidly and reversibly with CO_2 to form carbamino compounds, according to the following scheme [Faurholt, 1925, 2]:

$$R\mathrm{NH}_2+\mathrm{CO}_2 \rightleftharpoons R\mathrm{NH.COOH}, \qquad \text{.....(5)}$$

$$R\mathrm{NH.COOH} \rightleftharpoons \mathrm{H}^+ + R\mathrm{NH.COO}^-, \qquad \text{.....(6)}$$

$$\mathrm{H}^+ + R\mathrm{NH}_2 \rightleftharpoons R\mathrm{NH}_3^+. \qquad \text{.....(7)}$$

(The inhibitory effect of the proton is shown in this case also by the absence of reaction between CO_2 and $R\mathrm{NH}_3^+$.) The simultaneous occurrence of this carbamino reaction might make it difficult to detect and measure accurately any catalytic effect on the $CO_2+H_2O \rightarrow H_2CO_3$ reaction.

Cyclic nitrogenous bases have not, so far as we know, been investigated as regards their carbamino-forming power, and it was therefore with special interest that we tested some members of the new range of glyoxaline buffers, recently described by Kirby & Neuberger [1938], to whom we are greatly indebted for samples. Both the cations and the molecules of these substances were found to be incapable of any carbamino reaction with CO_2, but on the other hand the molecules proved to be markedly catalytic towards the reaction $CO_2+H_2O \rightarrow H_2CO_3$ especially if their pK were greater than 7·0. ($pK=-\log_{10}K$ where $K=[\mathrm{H}^+]\times[\text{molecule of base}]/[\text{cation of base}]$.) This led us to test such other cyclic nitrogenous bases as were both readily available and sufficiently soluble in water. Similar catalytic activity was always found if the pK were greater than 7, whereas tests of a more extended range of straight-chain N bases showed that these were without catalytic effect, their only reaction with CO_2, if any, being the carbamino formation. These rather remarkable results are summarized in Section II, together with some preliminary data on sulphhydryl and other compounds.

A possible mechanism of these catalyses is given in the discussion, together with some further points of interest which arise in regard to the enzyme carbonic anhydrase.

Methods

Rates of CO_2 uptake were determined by an improvement of Meldrum & Roughton's [1933, 2] manometric boat method. The apparatus (Fig. 1) consists of a lagged tank, a stationary manometer containing toluene coloured with sudan III as gauge liquid, a compensating bottle *E* (about 200 ml.), a CO_2 reservoir *D* (about 200 ml.) which can be connected through fine thermometer tubing *R* to a 60 ml. long-necked glass "boat" and a mechanism, not shown, for shaking the boat about *AR* as axis at an adjustable speed. The shaking must be rapid enough not to limit the rate of CO_2 uptake, but not so violent as to splash solution up into the tube connecting with the manometer. At 315 oscillations per min. the limiting speed is exceeded without the readings becoming erratic.

This speed is kept constant by a synchronous motor and is indicated by a neon lamp stroboscope.

The procedure in an experiment is as follows. The tank is emptied, and dried air is drawn through the connecting tubes to remove condensed moisture. In the meantime the test solution is measured into the "boat", which is stoppered and pushed into a holder in the shaker which holds it firmly by means of two rubber sleeves encircling the boat, all clips being open except C_4. The tank is then filled with water and the stirrer turned on. D is repeatedly evacuated and filled with CO_2 and left with a CO_2 pressure of 30–40 cm. Hg, the clips being closed and water-sealed. The boat, compensating bottle and manometer are evacuated to $\frac{1}{7}$ atm. through C_6 which is closed and placed below the water surface. After 9 min. the boat is shaken for 2 min. to bring the dissolved gases

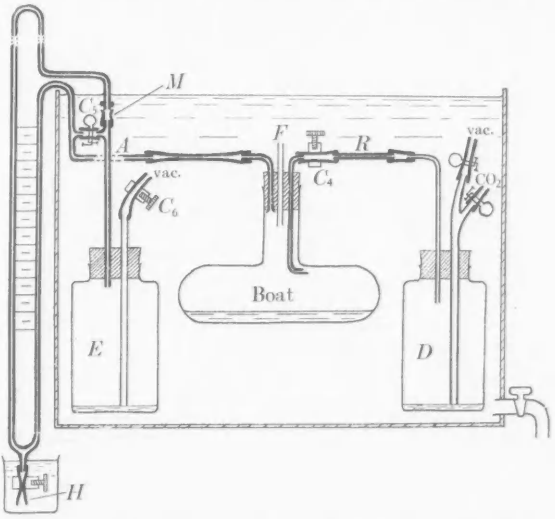


Fig. 1. Simplified diagram of apparatus arranged for CO_2 uptake. Not to scale. The boat is oscillated through 50° at right angles to the plane of the paper. Tube F is only used for anaerobic experiments.

in the test solution into equilibrium with the gas phase, and 1 min. is allowed for the solution to drain to the bottom of the boat. The short circuit C_5 between the two limbs of the manometer is closed and the zero reading taken. CO_2 is slowly let into the boat through the resistance R, by manipulation of C_4, up to the desired pressure. The time taken by this should be about 30 sec. A further period is allowed for the disturbance caused by the admission of the CO_2 to settle down, and then, exactly 2 min. after CO_2 begins to enter, shaking is started and manometer readings taken at 0, 5, 10, 15 sec. etc., up to 5 min. or more. The tank temperature is read. At the end of the experiment the clip C_5 is opened, and air allowed to enter slowly through a capillary inserted at C_6 until all parts of the apparatus are at atmospheric pressure. (If air enters suddenly into the apparatus, when at low pressure, the gauge liquid will shoot over.) Most of the water is meanwhile emptied out of the tank, and the boat then removed, washed and dried.

The preliminary equilibration period is 12 min.; if duplicate boats are used and one is prepared during the equilibration of the other, an observer and an assistant can perform serial experiments at the rate of one every 25 min.

With axial shaking, only a slight vibration is transmitted to the manometer and volume variations from distortion of the thick rubber tubing are negligible. Furthermore all the rubber joints can be water-sealed, the dead space between the boat and the liquid meniscus in the manometer cut down to 1 ml., and the volume of the system exposed to room temperature reduced to about 0·2 ml. These improvements, together with the substitution of toluene for water in the manometer gauge, the modified boat shape and the constant speed motor, are responsible for the increase in accuracy and reliability of the method. When the apparatus is working properly, readings should duplicate to 0·2 mm. At temperatures other than that of the room stirring of the water in the tank must be vigorously maintained during equilibration; otherwise the temperature, and therefore the pressure, in the compensating bottle or boat may vary from one another.

From time to time the whole apparatus must be washed out with water and acetone and dried by flushing with dried air. The manometer is then refilled with de-aerated coloured toluene through the rubber tube *H*. This tube is then filled with de-aerated water to form a seal and immersed in water. A little water is always left in *E* to saturate the gas with vapour.

When autoxidation, leaks or other complications which would give spurious results by manometric methods were suspected, readings were taken in absence of CO_2 during a prolonged first shaking period. For autoxidizable substances experiments were done under anaerobic conditions, by applying a slight modification of the method used by Meldrum & Roughton [1933, 2] for studying the rate of uptake of CO_2 by reduced blood to the present technique. If necessary one component of the required solution could be weighed solid directly into a dry boat, the oxygen removed by washing out with nitrogen and the remaining de-aerated solution added from a tonometer through *F*. In this case the substance makes no contact with oxygen whilst in solution.

Unless otherwise stated all experiments by the CO_2 uptake method were done at 0° with 4·2 ml. solution and an initial CO_2 pressure of 12·8 mm. Hg in a total gas space of 60 ml.

In order to avoid the effect of complications due to the $CO_2 + OH^- \rightarrow HCO_3^-$ reaction, most experiments were done below *p*H 8·0.

Rates of CO_2 output were determined in the same apparatus but with the double compartment boat method of Meldrum & Roughton [1933, 1]. After equilibration of the boat in the tank the solutions were suddenly mixed (by starting the shaker) and the CO_2 evolution followed manometrically. Most of the experiments were done at 0° and at a total gas pressure of $\frac{1}{5}$ atm.

The majority of the experiments were done with the illustrated apparatus which has however now been simplified. The outlet clip, water-seal etc., at *H* have been eliminated and the gauge fluid is introduced into the manometer tube through *M*.

Notes on solutions. Carbonic anhydrase was prepared in semi-purified state by Meldrum & Roughton's [1933, 1] chloroform method.

Buffer solutions were made up with a known acid/ion ratio by adding the calculated quantity of HCl or alkali, and checking the *p*H or titrating to an unbuffered end-point. The *p*H of solutions was determined in most cases colorimetrically but for special cases it was measured by the hydrogen electrode.

Na and K salts were used indiscriminately, except when high concentrations were required and one salt (usually K) is markedly more soluble than the other.

I. Oxy-acid buffers

The two buffers which have been most used hitherto in work on CO_2 kinetics are phosphate ($H_2PO_4^- + HPO_4^=$) and cacodylate ($HCac + Cac^-$), where Cac^- is

$$CH_3 \diagdown$$
$$CH_3 — As = O.$$
$$-O \diagup$$

We have accordingly investigated these with special care, and since the results and inferences therefrom seem qualitatively the same as for the general class of oxy-acid buffers which function above *p*H 6, we shall first describe the results with phosphate and cacodylate in detail.

CO_2 uptake experiments

Curve *A*, Fig. 2, shows the rate of CO_2 uptake by water, recorded manometrically. The gaseous CO_2 pressure drops to a steady value in about 20 sec.: this rapid uptake is simply due to physical solution of CO_2, the subsequent H_2CO_3 and HCO_3^- formation being negligible.

Curves *B*, *C*, *D*, Fig. 2, show the effect of increasing concentration of total phosphate, the ratio of $[HPO_4^=]$ to $[H_2PO_4^-]$ being kept constant. In each case there is a similar rapid drop of CO_2 pressure in the first 20 sec. (though the drop may be less owing to the solubility of CO_2 in the buffer being lower than in water), and then a prolonged slow phase in which the dissolved CO_2 enters into chemical combination. The slope of this slow phase is seen to be steeper

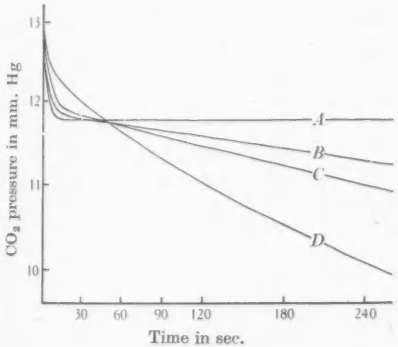


Fig. 2.

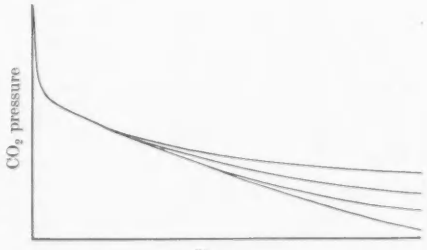


Fig. 3.

Fig. 2. Curves showing the observed course of CO_2 uptake by water and by phosphate at 0°. *A*, water; *B*, 0·0095 *M* phosphate; *C*, 0·14 *M*; *D*, 0·76 *M*. The *p*H was 7·4 at 0·2 *M*.

Fig. 3. Curves showing the expected course of CO_2 uptake by various concentrations of a given buffer, if the buffer itself has no direct effect on the CO_2 reaction. Corrected for effect of changing buffer concentration on physical solubility of CO_2.

throughout its *whole* course, as the phosphate concentration is increased; whereas if the phosphate did no more than just "instantaneously" remove the H^+ ions formed by the reaction $H_2CO_3 \rightarrow H^+ + HCO_3^-$, the slopes of curves *B*, *C*, *D* should be the *same* in their early stages, and the increased buffer concentration should exercise no effect until the latter part of the uptake, when the

velocity of the back reactions becomes significant, i.e. a family of curves like those shown in Fig. 3 should have been obtained.

Clearly there is, in addition, some direct effect of the phosphate upon the reaction, as is indeed shown quantitatively by equation (8) which fits the curves plotted in Fig. 2, i.e.

$$-\frac{d[CO_2]}{dt} = v_u [CO_2] = k_u [CO_2] \{1 + l_u [HPO_4^{=}]\}, \qquad \text{.....(8)}$$

where v_u is the apparent overall velocity constant for CO_2 uptake,
k_u is the true velocity constant of the reaction $CO_2 + H_2O \rightarrow H_2CO_3$,
l_u is a catalytic coefficient.

The numerical values of v_u in Tables I and III have been calculated by the method of Brinkman *et al.* [1933], but a correction has also been inserted for the rate of the back reaction, which in the case of phosphate amounts to 2–20 % and in the case of cacodylate to even higher figures. The allowance for the back reaction was made on usual principles and since it requires a knowledge of the end point of the reaction, this was determined either by continuing the experiment for 30 min. or preferably by a separate experiment in which a suitable amount of carbonic anhydrase was added to the mixture, so that the end point was reached in 5 min.

The time intervals over which v_u has been calculated have usually been 2 min. each, and in any given experiment at least four such intervals have been chosen between 1·5 and 8·0 min., v_u as given in Tables I and III thus being the average of four or more values. The individual figures in any one experiment usually agree with the mean to within 10 %.

A rougher, but less laborious, mode of calculation was used for the other data, which are mostly of a preliminary nature. This consisted in measuring the time t for the CO_2 pressure to drop through one or more small specified ranges in the early part of the slow phase of CO_2 uptake. Clearly $v_u = 1/t \times$ a constant, which is obtained from the curve of a solution of known v_u (determined as in Table I).

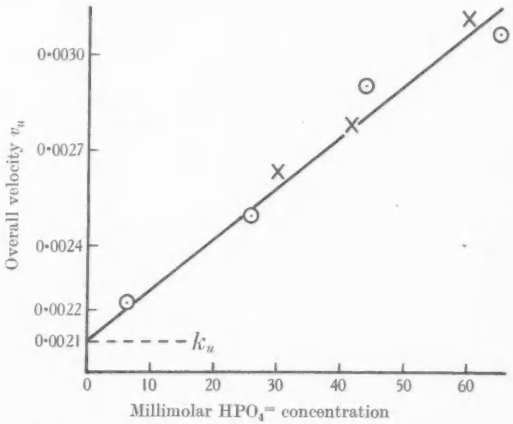


Fig. 4. Effect of $[HPO_4^{=}]$ on velocity of CO_2 uptake. ⊙ our experiments; × calculated from data of Brinkman *et al.* [1933].

Fig. 4 shows that there is a linear relation between v_u and $[HPO_4^{=}]$, at a ratio of $[HPO_4^{=}] : [H_2PO_4^{-}]$ of 2 : 1, as equation (8) requires. The corresponding

value of l_u, namely 8, together with l_u values for ratios ranging from 8 : 1 to 1 : 1 are given in Table I. The l_u values agree with one another within experimental error, thus demonstrating the validity of equation (8).

Table I. *Effects of phosphate and cacodylate on CO_2 uptake rate*

$[HPO_4^=] : [H_2PO_4^-]$	Conc. range (total phosphate, M)	l_u
8·0	0·01–0·20	7·7
3·2	0·01–0·20	8·0
2·0	0·01–0·10	7·7
1·0	0·01–0·10	8·5
	Mean	8·0

$[Cac^-] : [HCac]$	Conc. range (total cacodylate, M)	l_u
7·0	0·02–0·20	8·5
4·0	0·02–0·20	9·4
	Mean	9·0

A $[HPO_4^=] : [H_2PO_4^-]$ ratio of 1 : 1 was the lowest that could be used, for below this the CO_2 taken up becomes so small and the back reaction increases so fast that accurate determinations are not feasible. For the same reasons it was impossible to reduce the $[Cac^-] : [HCac]$ ratio below 4 : 1, for the pK of cacodylic acid is about 0·8 pH below the pK_2 of phosphoric acid, and even at 4 : 1 the back reaction corrections amount to as much as 30%, though in spite of this the v_u values over different intervals in any one experiment tally satisfactorily.

Above a total buffer concentration of 0·2 M, the physical solubility of CO_2 is appreciably depressed and the interpretation of the results thus becomes uncertain: in rough experiments, however, up to M it was found that v_u continued to increase nearly linearly with $[HPO_4^=]$. In the case of cacodylate v_u increased rather faster than $[Cac^-]$: this may be just an ordinary deviation from the law of mass action in strong solution, but it might also be due to some change in the state of the cacodylate at higher concentrations, such as the formation of a polymeride which is catalytically more active. A similar deviation occurs with chromate buffer solutions, in which there is known to be an equilibrium between the hydrochromate ion, $HCrO_4^-$, and the dichromate ion, $Cr_2O_7^=$, the amount of the latter increasing rapidly as the total chromate concentration is raised.

It was of interest to see whether equation (8) could also be applied to results published by earlier authors before the effect of the buffer was suspected. Adequate data are available in two such papers, and l_u values calculated therefrom are given in Table II. The excellent agreement with the present findings is a strong confirmation of the validity of our conclusions. Table II further shows that equation (8) is valid over a wide range of CO_2 concentration, namely 0·001 M (present paper) to 0·03 M (Faurholt).

Table II. *Values of l_u calculated from data in the literature*

Source	Buffer	CO_2, M	l_u	l_u from Table I
Faurholt [1925, 1, Table IV]	Cacodylate	0·03	9·5	9·0
Faurholt [1925, 1, Table VI]	Phosphate	0·018	8·4	8·0
Brinkman *et al.* [1933, Table III]	Phosphate	0·002	8·3	8·0

Extrapolation of the data, summarized in Table I (or in Table III [Brinkman *et al.* 1933]) to zero $[HPO_4^=]$ gives us for the first time an accurate value for k_u,

the true velocity constant of the reaction $CO_2 + H_2O \rightarrow H_2CO_3$. The mean value of k_u is 0·0021, i.e. about 25 % lower than the hitherto accepted value of 0·0027 [Table I, Roughton, 1935, 1], which is, of course, wrong, for in the calculations on which it was based no account was taken of the direct catalytic effect of the buffer.

Arguments against the effect being due to neutral salt action. It will be convenient now to exclude the possibility that the effect of the buffer may be accounted for by some kind of "neutral salt action" or ionic activity factor. This we do on the following grounds.

(*a*) The observed effects are larger than expected, and indeed are appreciable at concentrations, e.g. 0·01 *M*, much lower than those at which typical neutral salt action manifests itself. This holds not only for phosphate and cacodylate, but more so for the very active substances, such as selenite, tellurate and sulphite, to be dealt with later.

(*b*) Table III shows that addition of high concentrations of typical neutral salts, such as KCl and $NaNO_3$, to 0·04 *M* phosphate buffer (1 : 1), only increases the CO_2 uptake rate to a relatively slight extent, the l_u values for these salts being at most only one-sixteenth that of phosphate.

Table III. *Effect of neutral salts on the rate of CO_2 uptake by 0·04 M phosphate*

Salt	l_u
$(NH_4)_2SO_4$	0·2
$NaNO_3$	<0·05
$NaNO_2$	0
KCl	0·3
NH_4Cl	<0·05
KI	0·07
KCNS	0·5
$K_3Fe(CN)_6$	0·4

CO_2 output experiments

If the action of the buffer (or some accompanying impurity) is purely catalytic, then the reverse reaction $H_2CO_3 \rightarrow CO_2 + H_2O$ should be equally affected. On the face of it, this should be easy to test by measuring the rate of CO_2 output from mixtures of bicarbonate with varying strengths of buffer. Unfortunately, however, variation of the buffer strength will also affect the activities of the ions in solution, and may thus independently alter the rate of CO_2 output, which is directly proportional to the product of the H^+ and HCO_3^- activities. The exact nature of the possible "activity effects" is shown by the following treatment.

$$\text{The rate of } CO_2 \text{ output} = \frac{d[CO_2]}{dt} = v_o\,[H_2CO_3] = \frac{v_o a_H f_{HCO_3} [HCO_3^-]}{K_1}, \qquad \text{.....(9)}$$

where

v_o = overall output velocity constant,

a_H = hydrogen ion activity,

f_{HCO_3} = activity coefficient of HCO_3^-,

K_1 = true first ionization constant of H_2CO_3.

Now
$$a_H = \frac{K_2 f_{H_2PO_4} [H_2PO_4^-]}{f_{HPO_4} [HPO_4^=]}, \qquad \text{.....(10)}$$

where $f_{H_2PO_4}$, f_{HPO_4} = activity coefficients of $H_2PO_4^-$ and $HPO_4^=$ respectively,

K_2 = second ionization constant of H_3PO_4.

Therefore
$$\frac{d[CO_2]}{dt} = \frac{v_0 K_2 f_{HCO_3} f_{H_2PO_4} [H_2PO_4^-]}{K_1 f_{HPO_4} [HPO_4^=]} [HCO_3^-]. \qquad \text{.....(11)}$$

The activity coefficients of divalent ions, such as $HPO_4^=$, are much more affected, and in a more specific manner, than those of univalent ions, by changes in ionic strength, and there is no reason to suppose that the fraction $f_{HCO_3^-} \times f_{H_2PO_4^-}/f_{HPO_4^=}$ should remain constant as the phosphate concentration is reduced by dilution with water. But if the buffer is, instead, diluted with a mixture of K_2SO_4 and KCl, of the same total molarity as the highest concentration of buffer and with the same molar proportions of $SO_4^=$ to Cl^- as of $HPO_4^=$ to $H_2PO_4^-$ in the buffer, this difficulty should be practically overcome: for Landolt & Bornstein's Tables show that the activity coefficients of K_2SO_4 and Na_2SO_4 agree to within 0·01 with the activity coefficient of Na_2HPO_4 in pure solutions of the respective salts at the same concentration (up to 0·1 *M*), and it is therefore reasonable to suppose that in phosphate + sulphate mixtures the activity coefficients would be the same as in pure phosphate buffers of the same total molarity. A similar argument is applied to the Cl^- and $H_2PO_4^-$ ions.

All solutions, except bicarbonate, were freed from CO_2 by repeated evacuation and shaking. To compensate for the carbonate content of the bicarbonate solution, an equivalent amount of HCl was placed in the other solution used in the double compartment boat.

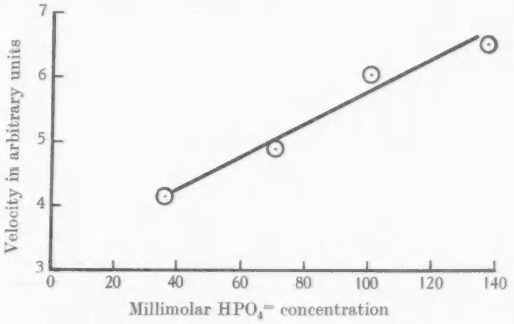


Fig. 5. Effect of $[HPO_4^=]$ on velocity of CO_2 output.

The methods of calculation were similar to those used in the CO_2 uptake work. Fig. 5 shows that there is again a linear relation between $[HPO_4^=]$ and v_o/K_1 and we may therefore write

$$\frac{v_o}{K_1} = \frac{k_o}{K_1}\{1 + l_o\,[HPO_4^=]\}, \qquad \text{.....(12)}$$

where k_o is the true velocity constant of the reaction $H_2CO_3 \rightarrow CO_2 + H_2O$, and l_o is the catalytic coefficient for CO_2 output corresponding to l_u for CO_2 uptake. Table IV gives the values of l_o for phosphate + sulphate + chloride mixtures and also for cacodylate + chloride ± acetate mixtures. It will be noted that in the case of both buffers l_o is independent of wide variations in *p*H and that the l_o values agree, within experimental error, not only with the l_u values of Table I but also with the l_u values given in Table IV, which were specially determined in buffer salt mixtures of the same composition as used in the output experiments. This last result proves that phosphate and cacodylate are both true catalysts of the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$.

The assumption that the ionic dissociation and reformation of H_2CO_3 are too fast to limit the rates of uptake or output of CO_2, even when the buffer

Table IV. *Effects of phosphate and cacodylate on CO_2 output rate*

	$[HPO_4^{=}]:[H_2PO_4^{-}]$	l_o	l_u
Phosphate + $SO_4^{=}$ + Cl^-	3 : 1	8·5	8·9
Phosphate + $SO_4^{=}$ + Cl^-	1 : 3	8·6	—
	$[Cac^-]:[HCac]$	l_o	l_u
Cacodylate + Cl^-	7 : 1	—	8·5
Cacodylate + Cl^-	2 : 1	9·2	—
Cacodylate + Cl^- + acetate	1 : 2	8·9	—

concentration is below $M/100$, perhaps requires some further words. Ionic reactions of this kind are generally assumed to be "instantaneous". A minimum value for their rate is given by rapid reaction velocity experiments such as those of Roughton [1930] which have shown that in presence of $M/10$ buffer the ionization of weak acids similar to H_2CO_3 has a half-period of less than 0·0003 sec. —probably far less. Whether decrease of buffer concentration would increase the half-period appreciably is very doubtful, since the ionization in complete absence of buffer, i.e. $H_2CO_3 + H_2O \rightleftharpoons HCO_3^- + H_3O^+$, is probably *per se* extremely fast. But, even if the rapid ionization of H_2CO_3 in buffer solutions were entirely due to a direct reaction with the buffer anion, i.e. $H_2CO_3 + A^- \rightleftharpoons HCO_3^- + AH$, a decrease of buffer concentration from $M/10$ to $M/100$ could not raise the upper limit for the half-period to more than $10 \times 0{\cdot}0003$ sec. $= 0{\cdot}003$ sec. Now calculation shows that, for the overall rate of CO_2 uptake in $M/100$ buffer solution to be retarded appreciably by the ionization of H_2CO_3, the half-period for the latter must be $> 0{\cdot}01$ sec. The action of the buffers cannot therefore be due to any effect on the ionic reaction $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$, but must be due entirely to their effect on the molecular reactions of CO_2 with water. Of several further points in favour of this we need only mention that the numerical values of k_u and k_o/K_1, as found by extrapolating to zero buffer concentration the respective rates of CO_2 uptake and output, check satisfactorily in two independent ways with the requirements of the law of mass action when applied to the molecular reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$. (Details will be given in a later paper by one of us.) If the rate of ionization of H_2CO_3 were of any limiting influence at low buffer concentrations no such checks would be obtained.

Additive test. An experimental comparison of the rates of CO_2 output from a mixture containing

(*a*) 0·017 *M* $NaHCO_3$ + 0·075 *M* K_2HPO_4 + 0·025 *M* KH_2PO_4 + 0·1 *M* KCac, with

(*b*) 0·017 *M* $NaHCO_3$ + 0·075 *M* K_2HPO_4 + 0·025 *M* KH_2PO_4 + 0·1 *M* Na acetate,

showed that the rate of (*a*) was 1·52 times that of (*b*). This demonstrates that the catalytic effects of the phosphate and cacodylate ions are additive, for on this basis we should have

$$\frac{\text{rate of } (a)}{\text{rate of } (b)} = \frac{1 + (8{\cdot}55)\,(0{\cdot}075) + (9{\cdot}05)\,(0{\cdot}1)}{1 + (8{\cdot}55)\,(0{\cdot}075) + (0{\cdot}6)\,(0{\cdot}1)} = 1{\cdot}49.$$

In this fraction 8·55, 9·05 and 0·6 are the respective l_o values of $HPO_4^{=}$, Cac^- and CH_3COO^- (Table IV).

The observed and calculated ratios agree much better than might have been expected from the size of the experimental errors.

Evidence that the catalytic action of the buffers is not due to an accompanying impurity. An impurity present in the salts and/or alkalis used in making the buffer solutions might be responsible for the catalytic effects observed.

The alkalis should first be considered, for they are used both by the manufacturer in preparing salts, such as KH_2PO_4, and by ourselves in making up the buffers. Two tests eliminate this possibility. (*a*) The effects are the same whether NaOH or KOH is used. (*b*) No increased effect is found when the buffer is treated with additional alkali if the latter is afterwards neutralized with an equivalent amount of HCl. It will be recalled that Cl^- itself does not inhibit—see Table III.

As regards the phosphate, we find exactly the same amount of catalysis whether all the phosphate solution is made up from KH_2PO_4 (Kahlbaum puriss. or AnalaR) or from Na_2HPO_4, $12H_2O$ (Kahlbaum puriss. or AnalaR). It seems unlikely that all these reagents would contain just the same amount of catalytic impurity. Furthermore since the effect is always proportional to the $[HPO_4^-]$ or $[Cac^-]$, the impurity would have to have the same *p*H-activity curve as both phosphate and cacodylate, the pK of which differ by 0·8. This is impossible if the impurities in the two cases are the same, whereas if there are two different impurities it would be a remarkable coincidence that their respective *p*H-activity curves should both happen to be identical with the ionization curves of the corresponding buffers.

Addition of sufficient carbonic anhydrase to double the rate of CO_2 uptake by $M/50$ phosphate, is also found to double the rate of CO_2 uptake by $M/5$ phosphate which, in absence of enzyme, it will be remembered, is nearly twice the rate of uptake by $M/50$ phosphate. The catalytic actions of carbonic anhydrase and phosphate thus appear to multiply one another instead of being merely additive, as would be expected if the phosphate catalysis were due to traces of an impurity. A possible mechanism for the multiplicative effect is put forward later.

That the effect can be due to metallic impurities is very unlikely because:

(*a*) Certain metals, notably Fe, are precipitated by phosphate at *p*H 8·0, yet the supernatant fluid from phosphate solutions which had been kept a long time showed the same effect as freshly prepared solutions.

(*b*) No increased effect was observed on adding to 0·04 M phosphate, *p*H 7·1, various possible impurities in traces, including many cations, e.g. Li^+, Cu^+, Cu^{++}, Ca^{++}, Sr^{++}, Ba^{++}, Mg^{++}, Hg^{++}, Pb^{++}, Fe^{++}, Fe^{+++}, Sb, La, U, Sn. The following cations had no (or very slight) additive effect on the rate of CO_2 uptake by cacodylate buffer, *p*H 6·8: Ca^{++} (0·2 M), Ba^{++} (0·2 M), Sr^{++} (0·2 M), Th^{++++} (0·001 M), La^{+++} (0·0005 M). At 0·0006 M, however, La^{+++} had a marked accelerating effect, but at this concentration a precipitate developed in the boat. 0·012 M Cu^{++} and 0·1 M Rb^+ in β-glycerophosphate, *p*H 6·8, had no effect.

(*c*) The rate of CO_2 uptake by phosphate, *p*H 7·1, is unaffected by adding 0·08 M cyanide.

This varied evidence, taken as a whole, makes it safe for the present to attribute the catalytic action in the main, if not entirely, to the more electronegative constituent of the buffer itself.

Further experiments on the nature of the catalysis

The natural question next arises as to whether this effect is limited to the cacodylate, the secondary phosphate and the borate ions (Faurholt's work) or whether it is shown by the more negative constituent of oxy-acid buffers in general. We have tested the matter by CO_2 uptake experiments on a wide range of buffers, either in pure solution or when mixed with phosphate buffer, in which case their additive effect, if any, on the rate of uptake by phosphate has been worked out. At *p*H > 9·0 results become less easy to interpret owing to the

appreciable intervention of the $CO_2+OH^- \rightleftharpoons HCO_3^-$ reaction, and we have therefore, for the present, restricted ourselves in the main to buffers with acid constituents of pK below 9·0. Our object being to make a broad preliminary survey of the whole field, we have in most cases contented ourselves with the rougher method of calculating l_u described above, and we have neglected any complications which may arise from the possible effects upon the solubility of CO_2 of certain of the substances which were used in high concentration: for these reasons we do not claim the same exactitude for these results as for those given in Tables I and IV, though we do believe that their order of magnitude is correct. The values of l_u, in terms of the more negative constituent of the buffer, are

Table V. *Effect of various oxy-acid buffers on rate of CO_2 uptake*

	Buffer	pK at 0°	[ion] : [acid]	[ion]+[acid],* M	l_u
I.	Phosphate	pK_2 7·1	—	—	8·0
	Phosphite	pK_2 6·7	2·0	0·20	6
	Pyrophosphate	—	—	—	10–50
	α-Glycerophosphate	6·4	10·0	0·40	4·5
	β-Glycerophosphate	6·3	3·0	0·25	3·0
	Phosphoglycerate	6·0	18·0	0·15	2·5
	Hexosediphosphate	6·3	9·0	0·23	8·5
	Cacodylate	6·3	—	—	9·0
	Arsenate	pK_2 6·8	2·0	1·20	6·0
II.	Maleate	pK_2 6·1	8·0	0·95	2·0
				1·00	1·6†
	Citrate	pK_3 5·4	c. 20·0	2·00	1·5†
	Veronal	8·0	3·0	0·18	8·0
III.	Chromate	pK_2 6·4	3·0	0·1	c. 50
	Borate	9·5	0·016	1·45	c. 150†
IV.	Sulphite	pK_2 7·0	3·0	0·1	900
	Selenite	pK_2 8·0	0·3	0·02	1700
				0·0036	>2000†
	Tellurate	pK_1 7·8	1·0	0·05	600
V.	Formate	3·8	—	1·0	0·5†
	Acetate	4·7	—	1·0	0·6†
	Phthalate	pK_2 5·3	—	0·8	0·8†
	Oxalate	pK_2 4·1	—	0·6	1·4†

* Highest total concentration used. † Measured in 0·04 M phosphate buffer.

given in Table V which shows clearly that the effect sought for is very widespread, being considerable with all oxy-acids of $pK > 6$, and in general increasing with pK. For convenience in discussion Table V has been divided into the following sections.

Group I consists of buffers closely related in structure to phosphate and cacodylate, and with pK in the range 6·0–7·0. Phosphite, with a pK_2 rather lower than the pK_2 of phosphate, has an l_u value also slightly less (i.e. 6·0 compared with 8·0). The organic phosphates with still lower pK have their l_u values correspondingly lower, except hexosediphosphate. But the molecule of this substance has two phosphate groups, each with pK at 6·3. Hence for calculating l_u, a M solution of hexosediphosphate should from the present point of view correspond to a 2·0 M solution of the ion. On this basis, l_u comes to be of the same order as for the other organic P compounds. Pyrophosphate shows considerable catalytic effect, but it is present both in the trebly and quadruply ionized forms under the pH conditions studied, and the respective catalytic contributions of these two forms have not yet been worked out.

Group II. Two organic acids, maleate and citrate, with pK near the lower limit at which activity is shown, have rather small l_u values. Veronal with a pK of 8·0 shows, as would be expected, a higher l_u value.

Group III. The two inorganic buffers chromate and borate have l_u values about 10 times greater than those in group I. In the case of chromate there is, as already mentioned, some uncertainty owing to the presence in solution of appreciable amounts of $Cr_2O_7^=$, besides the $HCrO_4^-$ and $CrO_4^=$ ions.

Faurholt [1925, 1] commented on the anomalous results found by him with borate even at pH 8·0, where complications arising from the $CO_2 + OH^- \rightarrow HCO_3^-$ reaction should only be slight. Inspection of his figures shows that the discrepancies to which he refers could be explained if we assign an l_u value to the borate ion of the order of 100–200—a reasonable figure in view of the pK of boric acid, i.e. 9·5. In borate-phosphate mixture we find an l_u value of about 150, thus confirming this suggestion.

Group IV. Sulphite, selenite and tellurate,[1] showed the greatest effects of any so far tested, l_u being in the neighbourhood of 1000. Careful controls showed that the results were not due to autoxidation. Their activity, being so much greater than that of other substances of similar pK, must be due to their special chemical constitution: in this connexion it is of interest to find this high activity associated with three elements of the same group (VI) of the Periodic Table. A further point of interest about the action of selenite will be discussed later.

The effects of chromate and selenite on CO_2 output were also investigated. The l_0 values in the two cases were found to be of the same order as the l_u values.

Group V contains some typical carboxylic acids with $pK < 5{\cdot}0$. Their l_u values, with the doubtful exception of oxalate, are all distinctly below 1·0. It thus appears that as the strength of the acid rises a range is reached (pK 6·0–5·0) in which the catalytic effect of the acid anion tends to fade out.

The effect of the bicarbonate ion

The l_u value for the HCO_3^- ion is not included in Table V, since it cannot be obtained either by the methods so far used in this paper or from data given in previous papers. It is, however, important to measure it, both from the point of view of the physico-chemical mechanism of the $CO_2 + H_2O \rightleftharpoons H_2CO_3$ reaction and also of practical applications to biological problems. Since the true first pK of H_2CO_3 is about 3·7 the l_u value for HCO_3^- would not, from Table V, be expected to exceed 1·0. The following modification of the usual technique shows that, in point of fact, the l_u value must be less than 0·5 and may be zero.

A 0·2 M $NaHCO_3$ in 0·004 M Na_2CO_3 mixture was prepared by dissolving the requisite weights of the two salts in recently boiled distilled water with minimal contact with air so as to reduce CO_2 exchange. 5 ml. of this solution were placed in the boat and shaken at 0° with the standard CO_2 pressure (in the usual way) for 90 min., i.e. until equilibrium was practically reached. The $[CO_3^=]$ was found to have increased to 0·005 M, and the $[HCO_3^-]$ to have dropped correspondingly. The pH of the solution was calculated to be 8·6. After 1 min. pause for drainage, a second vol. of CO_2 equal to the first was introduced into the boat in the usual way, and an ordinary experiment then carried out to completion. Still more $CO_3^=$ was formed and there was a further slight fall in pH. The overall velocity constant of CO_2 uptake was then calculated from the time course of the CO_2 uptake during the second shaking though far larger corrections were necessary than usual for (*a*) the velocity of the back reaction (50–60 %) and (*b*) the velocity

[1] Prepared from B.D.H. telluric acid. Two samples from other sources were unsatisfactory as they gave heavy precipitates when partially neutralized by alkali.

of the $CO_2 + OH^- \rightarrow HCO_3^-$ reaction which at *p*H 8·6 is about 30 % of the velocity of the $CO_2 + H_2O \rightarrow H_2CO_3$ reaction. Fortunately, these two large corrections are opposite in sign, and in consequence the final value of the overall velocity constant should be accurate to about ± 10 %. The average of four calculated values came out to 0·00208, in very close agreement with the value, 0·0021, given above for k_u, the true velocity constant of the reaction $CO_2 + H_2O \rightarrow H_2CO_3$. The joint catalytic effects of the HCO_3^- and $CO_3^=$ ions in this experiment thus cannot at most have exceeded 10 %, which means that l_u for HCO_3^- ion alone $> 0{\cdot}5$—a negligible value for practical purposes in work at $[HCO_3^-] < 0{\cdot}05$ *M*.

The experiment also gives an upper limit of about 25 for the l_u value of the $CO_3^=$ ion. Recent experiments at higher $[CO_3^=]$ suggest that the limit is almost certainly much lower.

II. Basic nitrogenous buffers and other substances

Basic nitrogenous buffers

We have tested three classes of N-containing bases: (*a*) compounds with the basic N in a closed ring, e.g. glyoxaline, nicotine, (*b*) compounds with the basic N in a straight chain, e.g. aniline and (*c*) mixed compounds containing both cyclic basic N and straight chain basic N, e.g. histidine. The results with class (*a*) being the most straightforward will first be described.

Cyclic N bases. Four members of the glyoxaline series and three other compounds were tested as regards their effect on CO_2 uptake by the methods described in Section I (see Table VI). Fig. 6 shows a typical pair of results for

Table VI. *Effect of cyclic nitrogenous bases*

Buffer	*pK* at 0°	[base] : [ion]	l_u	l_o
2:4(or 5)-Dimethylglyoxaline	8·80	0·33	12·5	—
4(or 5)-Methylgloxaline	7·97	2·0	9·5	—
		1·0	11	—
		0·5	9·5	—
		0·25	10	—
Glyoxaline	7·40	1·0	1·5	—
		2·0	—	*c.* 2
4(or 5)-Hydroxymethylglyoxaline	6·85	4·0	1·6	—
Nicotine	8·5 (8·0 at 25°)	0·5	13	—
Pilocarpine	7·30 (7·0 at 15°)	1·0	4·6	—
		1·0	—	5·0
Pyridine (in phosphate)	5·7 (5·3 at 20°)	50·0	<0·6	—

an equimolar buffer mixture of glyoxaline and glyoxaline hydrochloride at total concentrations of glyoxaline ranging from 0·036 to 0·38 *M*. It will be noted that the CO_2 uptake during the initial rapid phase is in both cases practically the same and is equal in amount to that expected from the solubility coefficient of CO_2, thus showing that in this case there is no rapid reversible combination of the base with CO_2 to form a compound of a carbamino type. (Contrast the results obtained in Fig. 7 with a typical straight chain N compound.) Nor was there any such indication in the case of any of the other compounds listed in Table VI.

When v_u, the overall velocity constant for CO_2 uptake, is calculated as in Section I and plotted against the concentration of unionized base (i.e. the more negative constituent of the buffer) a straight line is obtained. The slope of the

line shows that the l_u value for glyoxaline is about 1·6. Extrapolation of the line back to zero base concentration gives a value for k_u, the true velocity constant of the reaction $CO_2 + H_2O \rightarrow H_2CO_3$, in close agreement with that already found in Section I.

The l_u values given in Table VI have all been calculated in terms of the more negative constituent: the validity of this procedure was tested thoroughly in the case of 4(or 5)-methylglyoxaline by experiments at four different [N base : [N cation]] ratios ranging from 2·0 to 0·25. It is seen that l_u is constant within experimental error. It may be reasonably assumed that the other compounds in the Table would, on test, yield the same result.

Table VI also shows that there is a correlation between l_u and pK similar to that already found for the oxy-acid buffers in Table V (though in the present case the available range is smaller): thus, for example, the two compounds with $pK > 8{\cdot}0$, nicotine and 2:4(or 5)-dimethylglyoxaline, each have an l_u of about 12, whereas pyridine with a $pK < 6$ has a barely detectable l_u value. The compounds of intermediate pK have, broadly speaking, intermediate l_u values.

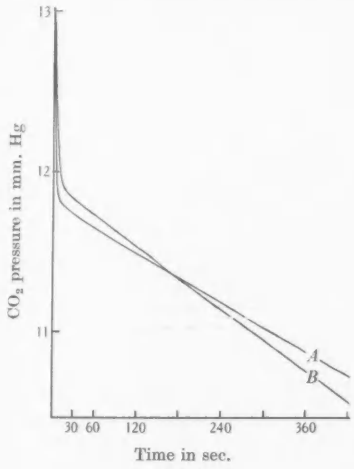


Fig. 6.

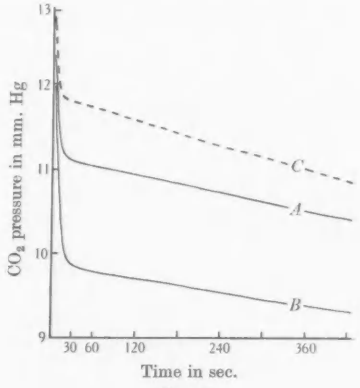


Fig. 7.

Fig. 6. Observed course of CO_2 uptake by glyoxaline. *A*, 0·018 *M* unionized glyoxaline + 0·018 *M* glyoxaline ion; *B*, 0·19 *M* unionized glyoxaline + 0·19 *M* glyoxaline ion.

Fig. 7. Observed course of CO_2 uptake by hydroxylamine buffer solutions. *A*, 0·036 *M* NH_2OH + 0·012 *M* NH_3 + OH^-; *B*, 0·085 *M* NH_2OH + 0·026 *M* NH_3^+OH/HPO_4^{--} + 0·011 *M*; *C*, 0·033 *M* $H_2PO_4^-$ for comparison.

Evidence for the catalytic nature of the effect was sought for as before, namely by comparing the l_0 values, obtained from CO_2 output experiments at different buffer concentrations, with the l_u values. Unfortunately, owing to experimental error and theoretical uncertainty, satisfactory accuracy can only be attained, if the l_0 value is > 5 and the $pK < 7$ (as is the case both for phosphate and cacodylate buffers). Of the compounds listed in Table VI pilocarpine approaches this ideal most nearly: the l_0 value from experiments over a range of 0·05–0·20 *M* total pilocarpine concentrations is 5·0, in satisfactory agreement with the l_u value of 4·6. A similar, but much less accurate test showed that the

l_o and l_u values of glyoxaline are of the same order of magnitude. The actual equation for the overall velocity constant of CO_2 output by N base buffers differs slightly from equation (11): it is

$$\frac{d[CO_2]}{dt} = \frac{v_o K_B f_{HCO_3} f_B [B^+]}{[B]} [HCO_3^-], \qquad \text{.....(13)}$$

where $[B^+]$, $[B]$ are the concentrations of N cation and base respectively.

K_B is the ionization constant of the base $= [B]\,[H^+]/[B^+]$.

f_B is the activity coefficient of the N cation.

The calculation of l_o in the case of N base buffers is thus much more sensitive to activity coefficient errors than in the case of the oxy-acid buffers, since a product of activity coefficients is involved instead of a quotient of activity coefficients as in equation (11).

Straight chain N bases. Faurholt [1925, 2] has measured the equilibrium constant K_{diss} for the carbamino reaction between CO_2 and the following straight chain amines: NH_3, CH_3NH_2, $(CH_3)_2NH$ and $CHNH_2COO^-$ (glycinate). As a measure of affinity of the bases for CO_2 we may take

$$1/K_{diss} = \frac{[CO_2]\,[B]^2}{[B^+]\,[carb]}, \qquad \text{.....(14)}$$

where [carb] is the concentration of carbamino compound formed. The values of $1/K_{diss}$ at 0° ranged from 10^3 to 10^6 indicating a very large affinity of these bases for CO_2. This, together with the fact that the pK value at 0° in all four cases is >10, makes it very difficult by the present methods to determine whether these bases have an appreciable l_u value, though Faurholt's work indirectly speaks against such a possibility.

We have, however, tested several straight chain bases of $pK < 9{\cdot}0$, and, in accordance with Faurholt's views, have found a much lower carbamino affinity for CO_2 so that it has been possible to allow for it fairly well in calculation of l_u. Typical results for hydroxylamine at a $[B] : [B^+]$ ratio of 3 : 1 and total concentrations 0·048 *M* and 0·11 *M*, are shown in Fig. 7.

The CO_2 uptake in the rapid phase is much greater than that taken up in physical solution (see control curve for CO_2 uptake by phosphate, and also Fig. 2) the excess, due to carbamino formation, is, as is to be expected from equation (14), proportional (at constant $[B] : [B^+]$) to the total hydroxylamine concentration. The data of Fig. 7 indicate that $1/K_{diss}$ for hydroxylamine is about 4, i.e. about 1/500 the value for ammonia, thus showing the marked effect of substituting an —OH group both on pK and on the affinity for CO_2.

It will be seen that the rate of CO_2 uptake during the slow phase is less in the 0·048 *M* hydroxylamine solution than in the phosphate control, and in the 0·11 *M* hydroxylamine solution less still. This is partly due to the lower pressure of CO_2 remaining in the gas phase and partly due to the fact that the $[HCO_3^-]$ in the hydroxylamine solution is formed not only from CO_2 coming from the gas phase but also from CO_2 dissociating from the carbamino compound, which is maximal at the end of the rapid phase and decreases as the solution becomes more acid [*v.* Roughton, 1935, 2]. Approximate corrections can be made for both of these effects: the former is obviously proportional to the extent by which p_{CO_2} is lower than the control, whilst the latter can be shown to be roughly equal to the $[carb]/[CO_2]$ in solution at the end of the rapid phase, provided that the [carb] is not too large. The carbamino corrections in the case of Fig. 7 amount to 7 and 20 % respectively and when applied, together with the p_{CO_2} corrections, show that the true rate of HCO_3^- formation in the hydroxylamine

solution is, within experimental error, equal to the basal rate in absence of catalyst, thus showing that hydroxylamine has no appreciable l_u value. Similar results were obtained with the other compounds listed in Table VII.

Table VII. *Straight chain N bases*

Compound	*pK*	Carbamino formation	l_u
Hydrazine	8·4 at 20°	+ + +	Undetectable
Hydroxylamine	6·2 at 20°	+ +	,,
Aniline	4·5 at 20°	+	,,
Acetamide	*c.* 0	0	,,
Urea	*c.* 0	0	,,

+ + + denotes that $1/K_{diss}$ is of same order as $1/K_{diss}$ for NH_3.
+ + denotes that $1/K_{diss}$ is of the order of 10^{-2} times $1/K_{diss}$ for NH_3.
+ denotes that $1/K_{diss}$ is of the order of 10^{-4} times $1/K_{diss}$ for NH_3.
0 denotes that $1/K_{diss}$ is less than 10^{-6} times $1/K_{diss}$ for NH_3.

Mixed compounds. From the above results we should expect that a compound which possesses both a basic cyclic N and a basic straight chain N should act both as CO_2 carrier and CO_2 catalyst. We have verified this in preliminary experiments on histidine, and two of its derivatives of physiological interest, dissolved in phosphate buffer. The results are shown in Table VIII.

Table VIII. *Effects of histidine and derivatives on CO_2 uptake rate*

Compound	*pK** at 22° Glyoxaline N	*pK** at 22° Side chain N	l_u	Carbamino formation
Histidine	6·15	9·3	*c.* 1·2	+ + +
Carnosine	6·8	9·5	*c.* 6	+ + +
Anserine	7·0	9·5	*c.* 3	+ + +

* Values and assignments from Deutsch & Eggleton [1938] who kindly supplied samples of anserine and carnosine.

Other buffers

We have also tested the catalytic effects of the anions of three non-oxy-acid weak acids, namely HCN (*pK* at 0° *c.* 10·0), H_2S (*pK* at 0° *c.* 7·0) and HF.

Cyanide. The l_u of CN^- was difficult to measure owing to the volatility and high *pK* of HCN. The following special technique was used. Scheele's HCN solution (4%, B.D.H.) was mixed with an equal volume of water in a burette over mercury, to avoid HCN loss by evaporation. Titration to *p*H 6·0 gave the mineral acid content of the solution (0·031 *N*), and further titration to *p*H *c.* 12 (Tropoeolin O as indicator) gave the HCN content (0·79 *N*). 4 ml. of this solution + 0·8 ml. of *M*/2 KCN were placed in the boat, the latter stoppered at once and the experiment carried out as usual except that the gas pressure was left at 1 atm. instead of being reduced. The standard amount of CO_2 was introduced from a reservoir at 19 cm. Hg positive pressure. The rate of uptake of CO_2 by this cyanide buffer mixture, which contains 0·66 *M* HCN and 0·06 *M* CN^-, was found to be about twice that of the basal rate below *p*H 7·5 in absence of catalyst. Fourfold dilution of the cyanide buffer in a second experiment showed a CO_2 uptake rate about 1·9 times the basal rate. The increase above the basal rate in the two experiments was mainly due to the rate of the $CO_2 + OH^-$ reaction, which in a 1 : 11 cyanide-HCN buffer mixture (*p*H *c.* 9·0) should be of the order of 80%[1] of the $CO_2 + H_2O \rightarrow H_2CO_3$ basal rate. If the residue of

[1] Exact allowance is difficult owing to uncertainty as to the *pK* of HCN at 0° and of the size of the appropriate activity corrections.

the increase is attributed to the catalytic effect of the CN^- ion, its l_u value comes out to be roughly 1·0 or less. CN^- therefore does not appear to belong to the catalytic cyclic N family, which at such a high pK should show an $l_u > 10{\cdot}0$. Nor does it form any carbamino compound, according to the present experiment and the previous ones of Meldrum & Roughton [1933, 2].

Sulphide. The pK_1 of H_2S at 0° is about 7·0 so that a catalytic effect of HS^- might possibly be expected. Unfortunately the high volatility of H_2S leads to appreciable loss of H_2S into the gas phase of the boat from the liquid as the latter becomes more acid during the CO_2 uptake. To minimize this, the $[HS^-]$ was kept down to 0·012 *M* by dissolving the requisite amount of NaHS (obtained by half-neutralizing Na_2S (A.R.) with HCl) in a buffer mixture containing 0·05 *M* 2:4(or 5)-dimethylglyoxaline base + 0·05 *M* dimethylgloxaline cation (*p*H *c.* 9·0). Calculation showed that, even so, a correction of 15 % must be inserted. The experiment was done in much the same way as the cyanide experiment and the uptake rate was found to be about 15 % slower than the control uptake rate by the same buffer mixture without added NaHS. Thus when the correction for H_2S volatility is applied the two rates agree within experimental error, showing that the catalytic effect of 0·012 *M* HS^- is inappreciable. The l_u value of the HS^- cannot therefore exceed 10·0 and may be zero.

The amount of CO_2 taken up during the rapid phase was found to be the same whether HS^- was present or not, thus showing no evidence of any appreciable rapid reversible combination between CO_2 and HS^- analogous to the carbamino reaction.

We have not yet tested any more complicated sulphhydryl acids, such as thiolacetic acid, owing to the higher pK of their –SH group (*c.* 10·0) and their instability.

Fluoride. The pK of HF is 4·45 at 25°, and the l_u value, calculated from an additive experiment with 0·6 *M* NaF + phosphate buffer, is 1·1. This value agrees closely with that of the oxy-acids of pK *c.* 4·0, and is distinctly higher than the range found for neutral salts of strong acids in Table III.

Discussion

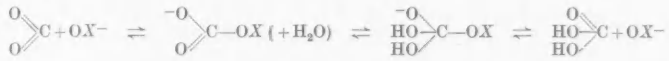
The mechanism of the catalysis

In the CO_2 output experiments the additional rate due to the catalysis is proportional to the product of the $[H_2CO_3]$ and the more negative constituent of the buffer, e.g. $[HPO_4^-]$ or $[Cac^-]$, and is independent of the $[HCO_3^-]$ except in so far as the latter conditions the $[H_2CO_3]$. This means that H_2CO_3, not HCO_3^-, is the substrate acted on by the catalyst, i.e. the reaction catalysed must be $CO_2 + H_2O \rightleftharpoons H_2CO_3$ and not $CO_2 + OH^- \rightleftharpoons HCO_3^-$. Preliminary confirmation of this has been obtained by CO_2 uptake experiments between *p*H 9·0 and 10·0 with two different $[H_2BO_3^-] : [H_3BO_3]$ ratios and varying total borate concentration. It was found that the fraction of the overall uptake rate due to the $CO_2 + OH^- \rightarrow HCO_3^-$ rate was practically unaffected by changes in $[H_2BO_3^-]$ although the fraction due to the $CO_2 + H_2O \rightarrow H_2CO_3$ was greatly affected by $[H_2BO_3^-]$, which showed its usual l_u value > 100.

Whilst not excluding the possibility of a chain mechanism we think it more likely that the catalysis can be explained by the intermediate compound formation between CO_2 and H_2CO_3 on the one hand, and the more negative constituent of the buffer on the other.

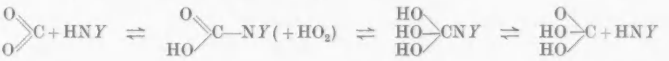
In the case of the oxy-acid buffers the compounds might be of a "carbonato"

type, analogous to the reversible compounds between CO_2 and organic hydroxides studied by Faurholt:



We have to suppose that H_2O can combine with the CO_2 bound to OX^- to form the corresponding $H_2CO_3^-.OX$ intermediate compound which in turn decomposes to $H_2CO_3 + OX^-$. We then have the catalytic scheme formulated in the equation. This scheme explains why (*a*) the reaction $CO_2 + OH^- \rightleftharpoons HCO_3^-$ is not catalysed; probably the electrostatic repulsion between HCO_3^- and OX^- hinders the formation of the necessary $HCO_3^-.OX^-$ intermediate compound, and (*b*) the catalytic activity of the oxy-acid ion tends to disappear when the pK of the acid is $< 6{\cdot}0$. Table VII shows how the affinity of the straight chain N bases for CO_2 declines *pari passu* with the decrease in pK, i.e. decreases in affinity for H^+. Similarly we might suppose that the combination of CO_2 with the $—O^-$ in weak acid anions persists up to a certain point as the strength of the acid increases but finally tends to become negligible. On this view the lack of catalysis is due to failure to form the necessary intermediate compound.

In the case of the cyclic nitrogenous base buffers the intermediate compound with CO_2 might be of a carbamino type:



With straight chain N compounds no reaction occurs between water and the carbamino compound of the N bases, but in the cyclic N compounds the postulated carbamino compound might, for some reason connected with the chemistry of the ring, tend to hydrate, forming the unstable intermediate which breaks down to H_2CO_3 and the free N base. The catalytic scheme is thus complete. Although at present we have no direct evidence in favour of these hypotheses, we have found them very useful working guides, and therefore have felt justified in mentioning them.

Additive effects

We have already alluded to the fact that the catalytic effects of $HPO_4^=$ and Cac^-, when jointly present, are additive and the same is also true of the following pairs—maleate + phosphate, chromate + phosphate and probably also borate + phosphate. Carbonic anhydrase and phosphate, on the other hand, tend to multiply the effects of each other. It was therefore of special interest to see whether our most active inorganic catalyst, selenite, is additive or multiplicative. It is difficult, owing to ionic activity uncertainties, to make such experiments fully satisfactory from a theoretical standpoint, but some preliminary results with selenite + phosphate, and selenite + chromate mixtures do suggest that in both cases selenite multiplies the effect of the weaker catalytic ion. Selenite may thus turn out to be a weak inorganic analogue of carbonic anhydrase, in which case a further detailed study of its catalytic mechanism would be well worth while.

As to the mechanism of the multiplicative effect it is possible that selenite and/or carbonic anhydrase may catalyse the formation of the intermediate compound between CO_2 and the more weakly catalytic anions, which we have supposed to be a preliminary to the catalysis and which may not itself proceed very rapidly. Various other explanations are however possible.

The effect of pH on carbonic anhydrase

As already hinted in the introduction, the original aim of the present research was to determine the effect of *p*H upon the activity of carbonic anhydrase.

Two difficulties were already obvious at the start, namely (*a*) in the *p*H range above 8·0, where CO_2 reacts in two ways (see equations (1) and (2)) there is the problem of sorting out the respective effects of the enzyme on these two different processes, and (*b*) the Michaelis constant of the enzyme is very high, and hence it is impracticable to work at substrate concentrations sufficient to saturate the enzyme—a prerequisite in *p*H-activity work. The results described in this paper, though at first sight adding yet a further complication, may on further investigation prove to simplify the study of the effect of *p*H on carbonic anhydrase. Thus in regard to (*a*) preliminary experiments suggest that carbonic anhydrase, like phosphate and other buffers, only catalyses the $CO_2+H_2O \rightleftharpoons H_2CO_3$, and does not affect the $CO_2+OH^- \rightleftharpoons HCO_3^-$. In regard to (*b*), it is already known from unpublished observations of the writers and others, that the activity of carbonic anhydrase increases markedly over the *p*H range 6·0–8·0. If it turns out that the activity of the enzyme in promoting CO_2 uptake at constant CO_2 pressure when plotted against *p*H gives a curve resembling the ionization curve of a weak acid, a result exactly analogous to the results with phosphate etc. would be obtained, and it might by further analogy be fair to infer therefrom the actual *pK* of the active group of the enzyme, even though the enzyme had not been saturated with substrate in the experiments. All this is perhaps too much to hope for, but at all events we have some valuable new pointers as to the investigation of the mode of action of carbonic anhydrase.

The catalytic effects herein described occur in many physiological buffers containing CO_2, and should therefore be borne in mind as possible factors in diverse physiological and biochemical processes and experiments. In particular, they may be of interest in bone equilibria, wherein carbonato-phosphate reactions of a type somewhat similar to that postulated in our catalytic schemes may take place, especially in regions of high local phosphate concentration.

Summary

1. Detailed improvements have been made in the manometric technique for measuring the rate of CO_2 uptake by solutions.

2. When CO_2 is taken up by buffer solutions the buffer substance itself has some direct effect on the reaction besides "instantaneous" removal of H^+ concerned in the ionization of H_2CO_3 to $H^+ + HCO_3^-$. Experimental proof is offered that the effect cannot be due to impurities in the solutions or to "neutral salt" action, but is proportional to the concentration of the more negative constituent of the buffer. Thus in the case of phosphate buffer, the overall velocity constant, v_u, is given by

$$v_u = 0{\cdot}0021\ [CO_2]\ \{1 + l_u\ [HPO_4^=]\},$$

l_u, the catalytic coefficient, being 8, and for cacodylate 9. The figure 0·0021 represents the true velocity constant of the reaction $CO_2+H_2O \rightarrow H_2CO_3$ and is about 25 % lower than the erroneous value previously accepted.

3. All other oxy-acids, so far tested, which buffer in the *p*H range 6–9 show similar effects, whereas salts of stronger acids have much smaller effects ($l_u < 1{\cdot}5$). The effects with sulphite, selenite and tellurate are much larger (l_u of order of 1000).

4. Straight chain N bases, e.g. NH_2OH, show no appreciable catalytic activity, though they combine readily with CO_2 to form carbamino compounds. Cyclic N bases, e.g. glyoxaline, on the other hand do not form carbamino compounds but do act catalytically. The effect is proportional again to the concentration of the more negative constituent—in this case the unionized N base. Values for l_u of the order of 10 were found for several cyclic bases of $pK > 7{\cdot}0$. With weaker bases the effect tends to disappear. Mixed compounds, e.g. histidine, show both catalytic action and carbamino formation.

5. Similar effects are observed on the rate of *output* of CO_2 from bicarbonate solution suddenly mixed with buffer, l_0 (output) for the oxy-acid buffers phosphate, cacodylate, chromate, selenite, and for the N base buffers pilocarpine and glyoxaline being equal to l_u. The effect must therefore be a catalysis.

6. It is shown that the buffers only catalyse the $CO_2 + H_2O \rightleftharpoons H_2CO_3$ reaction and not the $CO_2 + OH^- \rightleftharpoons HCO_3^-$ reaction. A mechanism is suggested in which CO_2 (or H_2CO_3) combines reversibly with the more negative constituent of the buffer.

7. The effects of phosphate and cacodylate are additive, but of the enzyme and phosphate are probably multiplicative.

8. Some biochemical implications of these results are discussed. They must be allowed for in work involving velocities of CO_2 reactions. Selenite is suggested as a possible inorganic model of carbonic anhydrase.

REFERENCES

Booth & Roughton (1938). *J. Physiol.* **92**, 36 *P.*
Brinkman, Margaria & Roughton (1933). *Philos. Trans.* **232**, 65.
Deutsch & Eggleton (1938). *Biochem. J.* **32**, 209.
Faurholt (1925, 1). *J. Chim. phys.* **21**, 400.
—— (1925, 2). *J. Chim. phys.* **22**, 1.
—— (1927). *Z. phys. Chem.* **126**, 72.
Kirby & Neuberger (1938). *Biochem. J.* **32**, 1146.
Meldrum & Roughton (1933, 1). *J. Physiol.* **80**, 113.
—— —— (1933, 2). *J. Physiol.* **80**, 143.
Roughton (1930). *Proc. roy. Soc.* A, **126**, 470.
—— (1935, 1). *Physiol. Rev.* **15**, 241.
—— (1935, 2). *J. Physiol.* **85**, 17 *P.*